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NERVE GROWTH FACTOR IN ALZHEIMER'S DISEASE: BIOLOGICAL EFFECTS AND THERAPEUTIC POTENTIAL

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Nerve Growth Factor in Alzheimer's Disease: biological effects and therapeutic potential

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Til Geira, Eyva, Gests og Heklu



Oh, where have you been, my blue-eyed son
And where have you been, my darling young one
I've stumbled on the side of twelve misty mountains
I've walked and I've crawled on six crooked highways
I've stepped in the middle of seven sad forests
I've been out in front of a dozen dead oceans
I've been ten thousand miles in the mouth of a graveyard
And it's a hard, and it's a hard, it's a hard, and it's a hard
It's a hard rain's a-gonna fall

From "A Hard Rain's a-Gonna Fall" by Bob Dylan

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder against which there is yet no disease modifying or curative treatment. Degeneration of cholinergic basal forebrain (CBF) neurons plays a role in the pathogenesis of AD and these neurons are highly dependent on nerve growth factor (NGF) for growth and survival. NGF has been proposed as a potential therapy for AD, but NGF does not pass the blood-brain barrier and must be delivered locally to the CBF to avoid side-effects. We tested targeted delivery of NGF to the CBF, using encapsulated cell biodelivery of NGF (NGF-ECB) in a first-in-man trial in AD patients. The primary objective was to examine safety and tolerability and the secondary objective to test for possible effects on cognition and biomarkers. Ten AD patients were implanted stereotactically with NGF-ECB implants targeting the CBF during 12 months (papers II-IV) or 6 months (paper V). Six patients were implanted with first-generation implants, which at implant retrieval showed a low NGF release. Following improvements of implants and cell survival, implantations of second-generation NGF-ECB implants were performed in four AD patients. The patients were monitored with regard to safety, tolerability and secondary outcome measures.

In **paper II**, we investigated CSF activity of the cholinergic marker choline acetyltransferase (ChAT) and found that in half of the patients, ChAT activity was significantly increased at 12 months of NGF delivery, compared to an age matched AD reference group. ChAT activity also correlated significantly with cognition, nicotinic binding and brain glucose utilization as well as brain atrophy and CSF biomarkers. In **paper III**, effects of the NGF delivery on EEG activity were investigated. A significant correlative pattern between alpha power and a) changes in cognition and b) CSF ChAT activity during the 12 month trial was found, indicating that the more stable cognition or increase in cholinergic activity, the more normalized the EEG activity. In **paper IV**, we investigated changes in brain atrophy on magnetic resonance imaging (MRI) over 12 months of NGF delivery and found that half of the patients showed less brain shrinkage, compared to an age matched AD reference group, and a better progression in clinical variables and CSF biomarkers. In **paper V**, in four AD patients implanted with second generation NGF-ECB implants for six months, we demonstrated safety and tolerability and at retrieval at six months, the implants exhibited high NGF release and good cell viability. All four patients could complete the study and no adverse events were deemed related to the implants or NGF delivery. Also in these four patients, a significant pattern of correlations between ChAT activity and cognition and nicotinic binding was shown. In **paper I**, we investigated levels of pro and mature neurotrophins in subjects with AD, mild cognitive impairment (MCI) and subjective cognitive impairment (SCI). Our results showed the presence of both pro and mature forms of NGF in human CSF. Increased proNGF levels in the MCI group were found compared to AD, and a pathological biomarker profile in CSF from MCI subjects was associated with higher proNGF levels.

In conclusion, our studies on NGF delivery in ten AD patients are the first cell therapy studies in the world demonstrating that implantation of encapsulated cells releasing NGF into the CBF in AD patients is safe and well tolerated. Stable cognition correlated with markers of increased cholinergic activity, indicative of a stimulation of the cholinergic system and a possible slowing of the neurodegenerative process.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers. They will be referred to in the text by their roman numerals:

- I. Hjorth E*, **Eyjolfsdottir H***, Zhu M, Cortés Toro V, Schultzberg M, Eriksdotter M. Forms of brain-derived neurotrophic factor and nerve growth factor in cerebrospinal fluid in mild cognitive impairment: relation to biomarkers and conversion to Alzheimer's dementia. *Submitted*. *shared first-authorship.
- II. Karami A*, **Eyjolfsdottir H***, Vijayaraghavan S, Lind G, Almqvist P, Kadir A, Linderöth B, Andreasen N, Blennow K, Wall A, Westman E, Ferreira D, Kristoffersen Wiberg M, Wahlund LO, Seiger Å, Nordberg A, Wahlberg L, Darreh-Shori T, Eriksdotter M. Changes in CSF cholinergic markers in response to cell therapy with NGF in patients with Alzheimer's disease. *Alzheimer and Dementia*. 2015; 11:1316-28. *shared first-authorship.
- III. **Eyjolfsdottir H**, Koenig T, Karami A, Almqvist P, Lind G, Linderöth B, Wahlberg L, Seiger Å, Darreh-Shori T, Eriksdotter M, Jelic V. Fast alpha activity in EEG of patients with Alzheimer disease is paralleled by changes in cognition and cholinergic markers during encapsulated cell biodelivery of nerve growth factor. *In manuscript*.
- IV. Ferreira D, Westman E, **Eyjolfsdottir H**, Almqvist P, Lind G, Linderöth B, Seiger Å, Blennow K, Karami A, Darreh-Shori T, Wiberg M, Simmons A, Wahlund LO, Wahlberg L, Eriksdotter M. Brain changes in Alzheimer's disease patients with implanted encapsulated cells releasing nerve growth factor. *J Alzheimers Dis*. 2015;43:1059-72.
- V. **Eyjolfsdottir H**, Eriksdotter M, Linderöth B, Lind G, Juliusson B, Kusk P, Almqvist O, Andreasen N, Blennow K, Ferreira D, Westman E, Nennesmo I, Karami A, Darreh-Shori T, Kadir A, Nordberg A, Sundström E, Wahlund LO, Wall A, Wiberg M, Winblad B, Seiger Å, Wahlberg L, Almqvist P. Targeted delivery of nerve growth factor to the cholinergic basal forebrain of Alzheimer's disease patients: application of a second generation encapsulated cell biodelivery device. *Alzheimer's Research & Therapy*. 2016;8(1):30.

OTHER PUBLICATIONS NOT INCLUDED IN THE THESIS

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
A β	β -amyloid 1–42
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADAS-Cog	Alzheimer's Disease Assessment Scale-Cognitive subscale
APOE4	Apolipoprotein E, genotype E4
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
CBF	Cholinergic basal forebrain
CDR	Clinical dementia rating
ChAT	Choline acetyltransferase
ChEI	Cholinesterase inhibitor
CSF	Cerebrospinal fluid
CT	Computerized tomography
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
FDG	¹⁸ Fluorodeoxy glucose
IADL	Instrumental activities of daily living
LP	Lumbar puncture
MCI	Mild cognitive impairment
MMP-9	Matrix metalloprotease-9
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
NGF	Nerve growth factor
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association
NMDA	N-Methyl-D-aspartate
P-tau	Tau phosphorylated at threonine 181
PET	Positron emission tomography
QEEG	Quantitative electroencephalography
SCI	Subjective cognitive impairment
T-tau	Total tau
Trk	Tyrosine kinase

PREWORDS

Rita Levi-Montalcini was born in 1909 in Turin, Italy to a wealthy family, her father was an engineer and mathematician and her mother an artist. Rita considered a career as a writer earlier on, inspired by the works of the Swedish author Selma Lagerlöf, but decided to study medicine at the University of Turin, from where she graduated with a medical degree in 1936. After her degree, she worked in research at the university. In 1946 she moved to the United States, to work at Washington University in St. Louis, planning for a temporary stay but she eventually became a professor at the University. She held a dual citizenship and lived and worked in the United States for 30 years.



In her lab, she observed that an animal tumor that had been grafted onto a chicken embryo showed an increased nerve growth. Levi-Montalcini then adapted the experiment and placed the tumor so that it would share blood supply with the chicken embryo, after which she saw the same increase in nerve growth. Thereafter she repeated her experiments with nerve tissue and came to the same conclusions. She began working with the biochemist Stanley Cohen, and together they eventually isolated nerve growth factor (NGF), a protein that stimulates nerve cell growth and survival.

Although the scientific community did not initially appreciate the importance of NGF, their discovery of its importance became acknowledged with time and Levi-Montalcini and Cohen were awarded a Nobel Prize in Physiology or Medicine in 1986.

In 1962, Levi-Montalcini helped establish the Institute of Cell Biology in Rome and in 1992 she created an educational foundation and later on set up the European Brain Research Institute in 2002. Levi-Montalcini continued conducting research toward the end of her life. She died in Rome on December 30th, 2012 at the age of 103, thereby becoming the longest living Nobel Laureate.

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Rita Levi-Montalcini-Biographical. Available from: <http://www.Nobelprize.org>

1 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

Dementia is defined as a constellation of symptoms of cognitive decline impairing an individual's ability to live a normal life. Alzheimer's disease (AD) is the most common cause of dementia, representing 60-80% of dementia cases in Europe and North America (1, 2). AD is a progressive neurodegenerative disorder causing a successive decline in cognitive functions over years, eventually leading to death. Dr. Alois Alzheimer described in 1907, the neuropathological and clinical features of a female patient who had died of a mental illness that caused her to suffer from memory loss, language impairment and behavioral changes (3). After her death, dr. Alzheimer found clumps and tangled bundles of fibers on a brain autopsy, now named amyloid plaques and neurofibrillary tangles, respectively (4). The plaques and tangles are still considered among the cardinal features of AD pathophysiology.

1.1.1 Epidemiology

AD is the most common dementia disorder in older people and is a growing epidemic across the world. In 2015, the World Alzheimer Report described a dementia prevalence of around 47 million people worldwide, and estimates this number to increase to 131 million by 2050 (5). Worldwide, over 9.9 million new cases of dementia are diagnosed each year and an increased proportion of new cases arise in Asia, the Americas and Africa, while studies from Europe have reported decreasing incidence (6). The global costs of dementia have increased by over 35%, from 2010 to 2015, representing around 1.09% of global gross domestic product (GDP) including informal care, and total direct costs are estimated to be around 0.65% of global GDP (5). In Sweden, approximately 100 000 patients are afflicted by AD (7) and costs in Sweden are estimated to be about 63 billion Swedish kronor (8).

1.1.2 Risk factors

Risk factors for AD can be divided into modifiable risk factors and non-modifiable genetic risk factors, but the most significant risk factor for AD is increased age (9).

Genetic risk factors for AD include having an E4 allele of the APOE gene (APOE4), which is considered to be the major genetic risk factor for AD, where both homozygosity and heterozygosity pose an increased risk for AD (10, 11). Mutations in amyloid precursor protein (APP), and the presenilin-1 and presenilin-2 genes cause an autosomal dominant early onset of AD (12) and the triggering receptor on myeloid cells 2 (TREM2) allele causes

a rare genetic predisposition to AD (13). Genome-wide studies have furthermore identified more than 20 loci associated with an increased risk of AD, pointing at immune pathways and inflammatory responses, endosomal-vesicle recycling and lipid metabolism (14, 15).

Cardiovascular risk factors such as diabetes mellitus (DM), hypertension, obesity and smoking and psychosocial factors such as low education and low mental activity are thought to be the main modifiable risk factors for AD and targets for intervention (16). Based on findings from the Rotterdam population study, it has been hypothesized that elimination of modifiable risk factors would lead to a reduction in dementia incidence by as much as 30% (17). Studies have also shown that having the APOE4 genotype may augment both the risk of environmental risk factors and the severity of chronic diseases like DM, hypertension, hypercholesterolemia, depression as well as low physical and cognitive activity (18, 19). In addition, traumatic brain injury is considered to be a significant environmental risk factor for AD (20, 21), most likely induced both by chronic inflammation within the brain as well as increased levels of amyloid beta protein (A β) (22, 23).

1.1.3 Pathophysiology

Even though studies have provided evidence for multicausality of AD, there is convincing evidence for the involvement of plaque accumulation, due to abnormally folded A β , and neurofibrillary tangles comprised of phosphorylated tau protein (12), in neurodegenerative processes in the brain (24). Studies on patients suffering from a familial form of AD, caused by mutations in the APP, PSEN1 or PSEN2 genes, provide evidence further linking A β to the pathophysiological cascade in AD (25). APP is the precursor of A β and mutations in the APP gene affect the cleavage of A β , while those in the PSEN1 and PSEN2 genes affect γ secretases, which cleaves APP, causing a generation of longer and more hydrophobic A β peptides (26). Tau gene mutations on the other hand lead to a development of frontotemporal dementia (27). Previously, tau pathology was thought to be downstream of A β pathology, but it has lately been hypothesized that A β and tau pathologies in the brain occur in synergy (28) (*figure 2*).

1.1.4 Clinical features and diagnosis

Results from prospective studies point out the loss of episodic memory (memory of autobiographical events e.g. time, places), as the most robust neuropsychological predictor of dementia development (29, 30), which can be detected years before dementia diagnosis (31). Loss of episodic memory has also been shown to be associated

with increased amyloid burden, as demonstrated by a recent meta-analysis (32). Lower premorbid episodic memory also seems to be a predictor of developing dementia (33, 34). In addition, the presence of an APOE4 allele seems to moderate dementia development in subjects with low episodic memory, although reports are not entirely consistent on this matter (35). Impairment of semantic memory (memory of facts and events) can also be a prominent early clinical symptom in AD. Both semantic and episodic memory functions are processed in the medial temporal lobe albeit in different subregions. Traditionally, it is suggested that early signs of pathology can be seen in the transentorhinal cortex (moderating semantic memory), and thereafter the entorhinal cortex and hippocampus regions (36) (moderating episodic memory). Longitudinal studies have reported semantic memory impairment years before dementia onset, in parallel with hippocampal atrophy and A β pathology (37). Impairment of executive functions (e.g. attention, inhibitory control, working memory, cognitive flexibility, reasoning, problem solving and planning), processed by the prefrontal cortex (38), occur in the early stages of AD but there is growing evidence supporting that executive dysfunction may also occur in the preclinical stages (39).

AD has a progressive nature and the progress is often divided into mild, moderate and severe stages after the onset of dementia syndrome. As the disease progresses with time, additional cognitive functions become affected such as speech, orientation and motor planning, leading to aphasia and apraxia. Behavioral and psychiatric symptoms may develop, such as depression, mood instability, restlessness and disturbance in circadian rhythm. In the severe stage of AD, the patients have lost the ability to carry on a conversation, respond to their environment and eventually control movement and require extensive assistance with activities of daily living. They become vulnerable to complications of immobility and may suffer infections, blood clots and pressure wounds.

The gold standard for establishing an AD diagnosis is a brain biopsy or autopsy of the post-mortem brain, displaying the presence of characteristic histopathological signs of AD. However, brain biopsy is not often performed in clinical praxis, due to its invasive nature. Commonly applied clinical criteria for diagnosis of AD are the International Classification of Disease 10th revision (ICD-10) (40), Diagnosis and Statistical Manual of Mental Disorders 4, text revision (DSM-IV, TR) (41) and the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS), and the Alzheimer's Disease and Related Disorders Association (ADRDA) workgroup criteria (42). More recent diagnostic criteria, developed by the International Working Group (IWR) and the National Institute on Aging and Alzheimer's Association (NIA-AA) support the inclusion of cerebrospinal fluid (CSF) and imaging biomarkers (43) to the criteria.

1.1.4.1 Mild cognitive impairment

The term mild cognitive impairment (MCI) is used to describe a syndrome of cognitive impairment greater than can be expected for age and education level, that does not significantly interfere with activities of daily living (44). MCI can affect memory function (amnesic MCI), memory plus another cognitive domain e.g. language (multi-domain amnesic MCI), single non-memory domain (non-amnesic MCI) or multiple non-memory domains (multidomain non-amnesic MCI) (45). MCI is often a transitional state between normal cognition and clinically probable AD. Individuals with MCI are a heterogeneous group with an overall increased risk for developing dementia (46, 47), but not all cases of MCI have a neurodegenerative etiology. Longitudinal community-based studies have reported an incidence of 5-10% among MCI patients, but reported incidence of dementia among MCI subjects from specialty clinics is higher, or 10-15% (48).

Recommendations for general criteria for MCI, Winblad et al, 2004 (49):

[Cognition] not normal, not demented (does not meet criteria (DSM IV, ICD-10) for a dementia syndrome)
Cognitive decline: -Self and/or informant report and impairment on objective cognitive tasks -Evidence of decline over time on objective cognitive tasks
Preserved basic activities of daily living / minimal impairment in complex instrumental functions

1.1.4.2 Subjective cognitive impairment

Subjective cognitive impairment (SCI), recently often referred to as subjective cognitive decline (SCD), is common in an aging population. SCI is generally defined as complaints of cognitive decline in the absence of objective evidence of cognitive impairment on neuropsychological testing (50). The clinical picture is thus less advanced than in MCI, where the cognitive impairment can be confirmed using cognitive testing tools. There is increasing evidence supporting that individuals with SCI are at an increased risk for developing an abnormal CSF biomarker profile and have an increased risk for further cognitive decline in the future and development of AD dementia (51-53).

1.1.5 Predictive and diagnostic biomarkers for AD

A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention” (54). The National Institute on Aging-Alzheimer’s

Association (NIA-AA) has proposed the use of a panel of CSF and imaging biomarkers to determine the probability of AD (55) and risk assessment in prodromal stages of AD (56, 57).

Research biomarkers for AD are divided into three categories, each corresponding to pathophysiological changes in the brain (58):

- biomarkers of A β deposition:
 - low A β in CSF (59),
 - amyloid positron emission tomography (PET) scan shows high tracer retention (60),
- biomarkers of tau pathology:
 - increased levels of phosphorylated tau at threonine 181 tau (p-tau) in CSF,
 - increased tracer retention on the recently developed tau PET (61),
- biomarkers of neuronal degeneration:
 - increased levels of total tau protein (t-tau) in CSF,
 - diminished brain glucose utilization on ¹⁸F-fluorodeoxyglucose (FDG)-PET,
 - atrophy of brain structures characteristically affected by AD on magnetic resonance imaging (MRI).

There is convincing evidence suggesting a sequence of pathological events in the brain, eventually leading to cognitive decline and dementia. Earliest are signs of amyloid deposition (detected by low CSF A β and pathological amyloid PET), thereafter signs of tau pathology and neurodegeneration (detected by CSF p and t-tau levels, FDG-PET, atrophy parameters on MRI), followed by cognitive impairment (62). There is however not a clear association between magnitude of amyloid and tau pathology and clinical severity, illustrated by the disappointing results of phase III clinical trials with monoclonal antibodies targeting A β , which have shown a reduction in biomarker pathology but failed to demonstrate a clinical effect (63). Recent data suggest that tau accumulation, assessed with tau PET, may correlate more closely to clinical status than A β deposition (64) and this observation is supported by recent promising results from a phase I clinical trial testing tau vaccination in AD patients during 22 months (65).

In clinical datasets, low CSF A β , high t-tau and p-tau, provide a sensitivity of 82-86% and a specificity of 61-87%, for an individual pathological biomarker for differentiating AD from controls, and an even higher sensitivity and specificity is attained by calculating a t-tau/A β ratio (66). There is however a large variability between laboratories in biomarker measurements that has hampered efforts to define uniform reference intervals. This is caused by differences in enzyme-linked immunosorbent assay (ELISA) methods between laboratories and variability in reagents and manufacturing procedures, resulting in

variations from batch to batch, most pronounced for A β (67), but standardization work is ongoing.

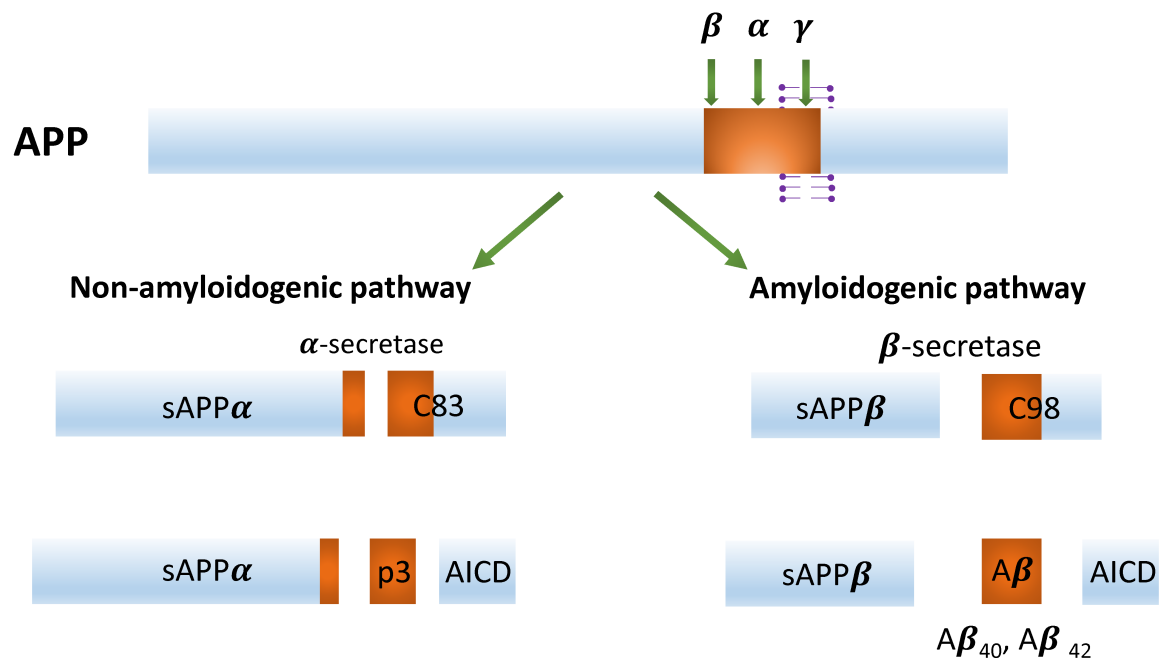
1.1.6 Current treatment for AD

Current available treatment focuses on targeting the early cholinergic dysfunction in AD (68) by inhibiting acetylcholinesterase, with a drug group named cholinesterase inhibitors (ChEI). ChEIs do however not reverse the progression of AD but provide only a symptomatic relief (69, 70). Donepezil may however increase the clearance of A β (71). ChEIs can stabilize cognitive outcome and daily function during the first year, but further cognitive and functional impairment occurs (72). Memantine, a non-competitive N-Methyl-D-aspartic acid (NMDA) receptor antagonist, is the only approved drug besides ChEIs for AD and it may provide some benefit for patients with moderate to severe dementia, as monotherapy or in combination with ChEIs. Swedish National Guidelines for Care in cases of Dementia recommend ChEI for treatment of mild to moderate AD and memantine for moderate to severe AD (7). For behavioral symptoms, non-pharmacological actions are recommended initially, followed by psychotropic drugs if necessary. Supportive care from caregivers and family is also important. Due to the multicausality of AD, possible therapeutic targets are many and new treatment strategies are needed urgently.

1.2 AMYLOID HYPOTHESIS AND LINKS TO TAU PATHOLOGY

The cleavage of APP by β - and γ -secretases results in formation of 39-43 amino acid A β peptides (73), which are secreted into the extracellular space. A major part of A β is secreted as the A β_{40} isomer, which is thought to have less tendency to aggregate (74). A β_{42} is more prone to form oligomers, protofibrils and fibrils and is the main A β isomer found in amyloid plaques (75, 76). A β_{42} has toxic effects on synapses, particularly in the postsynaptic compartment (77), resulting in impairment in long-term potentiation (78). A β_{40} and A β_{42} can be measured in CSF and both A β_{42} and the A β_{42} : A β_{40} ratio have been shown to be decreased in CSF from AD patients.

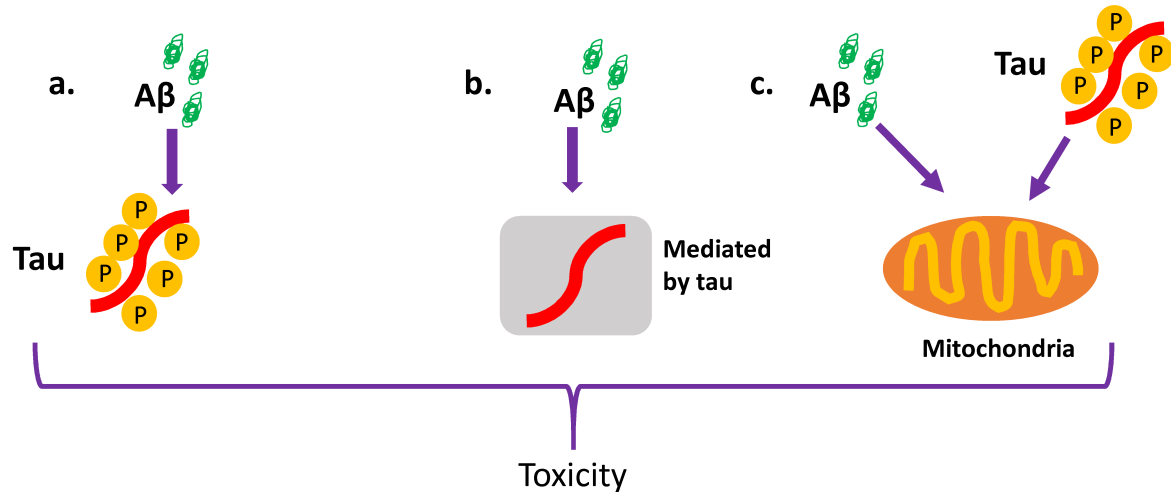
Figure 1. Non-amyloidogenic and amyloidogenic pathways of APP processing. A β isomers A β_{40} and A β_{42} are produced from the amyloidogenic cleavage of APP via the β -secretase (BACE1) and γ -secretase complexes. The non-amyloidogenic pathway via the α -secretase and γ -secretase complexes abolishes A β production. Modified from Del Prete et al, 2014 (79).



The amyloid cascade hypothesis, which has been the reigning dogma in AD research for the last decades, is a “linear, and quantitative model” (3), assuming direct cause and consequence of A β deposition, leading to tau pathology and subsequently AD pathology in the brain with inflammation, synaptic dysfunction and neuronal loss. The quantitative aspects of the hypothesis assume that the more amyloid plaques or A β deposition, the more pathology, but this remains controversial (80). Moreover, the association between magnitude of neuropathological changes and clinical severity is rather weak, as demonstrated by the failure of clinical trials, which have shown a partial clearance of A β plaques but failed to demonstrate clinical efficacy (81).

Hyperphosphorylated tau interferes with neuronal function even before its aggregation, through interference with axonal transport and mitochondrial respiration (82-84). It remains unclear where to place tau in the A β cascade hypothesis, whether its pathology is upstream or downstream of A β pathology or a parallel process, and evidence suggests multiple modes of interaction between A β and tau pathologies (85).

Figure 2. Possible modes of interaction between A β and tau pathologies: a. A β upstream of tau pathology, inducing hyperphosphorylation of tau. b. A β toxicity dependent on the presence of tau mediation and c. A β and tau pathologies as parallel processes attacking cell organelles in synergism. Modified from Ittner et al, 2011 (85).

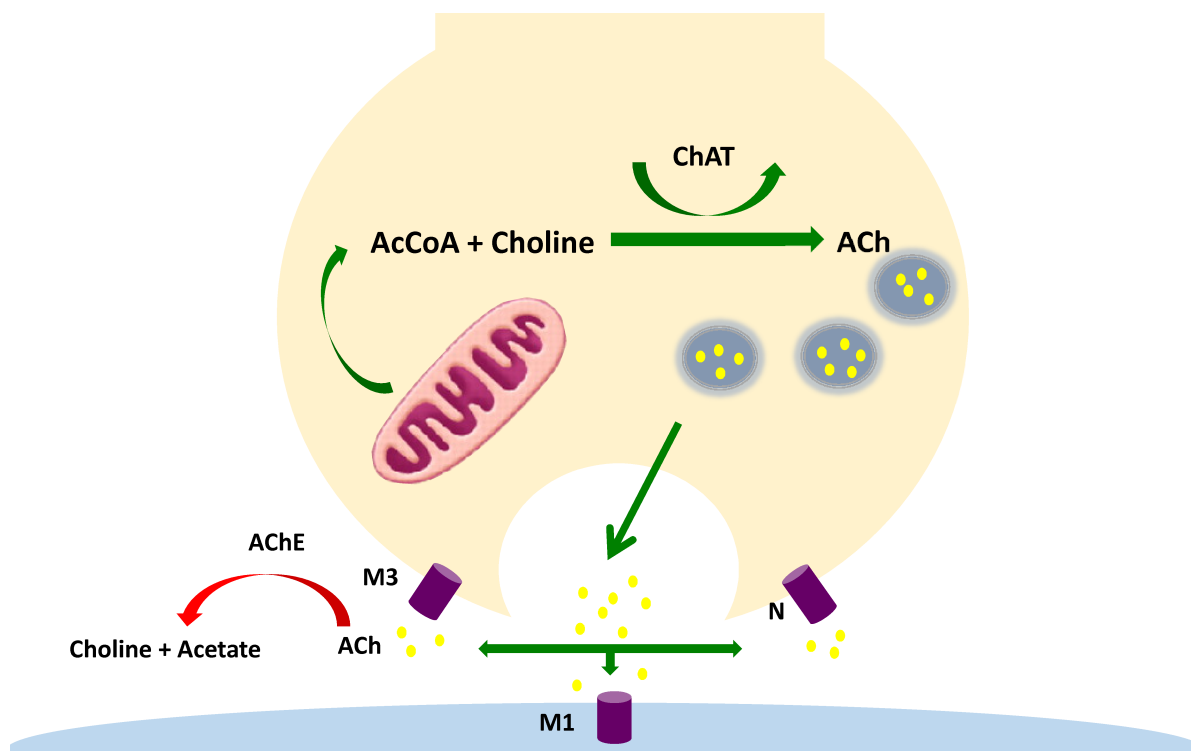


1.3 CHOLINERGIC BASAL FOREBRAIN AND ITS ROLE IN AD

The cholinergic basal forebrain (CBF) is comprised of the septal nuclei (Ch1), the vertical (Ch2) and horizontal limbs (Ch3) of the diagonal band of Broca and the nucleus basalis of Meynert complex (Ch4). The CBF provides major cholinergic projections to the cerebral cortices, hippocampus and amygdala (86, 87). Studies have shown that the CBF system has a highly organized topographic structure of efferent projections providing the major source of cholinergic innervation to the cerebral cortex (87-90). There is a bulk of evidence from studies on animals and humans suggesting the importance of the cholinergic system in cognitive functions such as learning (91, 92), memory (93) and attention (94). In AD, there is an early specific degeneration of the cholinergic synapses in the basal forebrain, which correlates highly to the cognitive loss (95, 96), severity of cortical synapse loss and density of amyloid plaques (97). Interestingly, it was recently demonstrated that histopathological changes in the cholinergic basal forebrain (CBF) precede changes in the entorhinal cortices (98). This cholinergic hypothesis for the pathogenesis of AD laid the ground for the development of the ChEIs, which inhibit the activity of acetylcholinesterase, the synaptic acetylcholine (ACh) degrading enzyme, thus increasing synaptic ACh levels. As the disease progresses, ChEIs' effect become insufficient in halting cholinergic dysfunction, perhaps due to the progressive loss of synapses releasing ACh (99) and severe loss of CBF neurons in advanced AD (100).

Cholinergic neurons are defined by the intracellular presence of the ACh synthesizing enzyme choline acetyltransferase (ChAT), and ChAT is thus regarded as the most selective cholinergic marker. ChAT synthesizes ACh from Acetyl-CoA and choline in the cell cytoplasm, which then is transported via synaptic vesicles and after exocytosis released into the synaptic cleft, where it binds to its muscarinic or nicotinic ACh receptors. ACh at the synaptic cleft is degraded by hydrolysis via acetylcholine esterase (AChE) (101).

Figure 3. Schematic representation of ChAT synthesis in the cytosol, ACh transport and degradation in the synaptic cleft.



Researchers have shown that the transcription of ChAT is diminished in the remaining cholinergic neurons, leading to decreased ChAT activity in AD (102, 103). Postmortem tissue from AD brains show signs of cholinergic impairment, including reduced ChAT activity, and diminished ACh release has been reported in biopsies from patients with mild AD (104). Cholinergic loss in AD correlates furthermore well with impairment of memory and attention (102, 105).

Acetylcholine receptors

ACh receptors are classified as either muscarinic or nicotinic, according to agonist

selectivity and pharmacological functions (101). Nicotinic ACh receptors are ion-gated receptor channels, selective for cations (106), generating a rapid response following activation. Nine types of nicotinic receptors have been identified so far. Different types of receptors are expressed in different organs of the body e.g. the central nervous system, peripheral nervous system and muscles and each type of receptor has distinct properties (107). Neuronal nicotinic ACh receptors are comprised of a combination of α or β subunits (108) and the most widely expressed subunit is $\alpha 7$ (109), composed of seven nicotinic α -subunits. Muscarinic ACh receptors are G-protein receptors leading to activation of second messengers. Genes for five isoforms of muscarinic receptors have been identified, M1, M2, M3, M4 and M5 (110), which can modulate various ion channels and can promote either cell excitability or voltage inhibition leading to diminished cell excitability (101).

Function and survival of the CBF neurons is highly dependent upon the neurotrophin nerve growth factor (NGF), retrogradely transported from the hippocampus and cortex (111). It has moreover been suggested that loss of neurotrophic support to the CBF may precede degeneration of the CBF neurons (112). A wealth of data from animal models, indicate that NGF effectively decreases cholinergic deficit and cognitive impairment (113).

1.4 NEUROTROPHINS

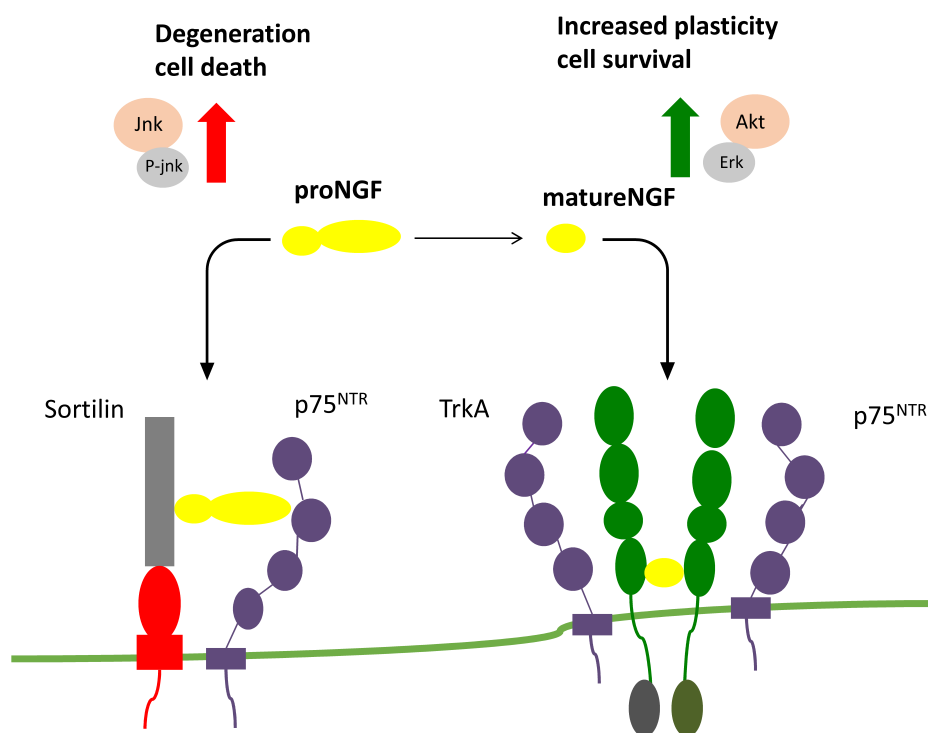
Neurotrophins comprise a family of structurally related peptides that regulate differentiation, growth and survival of neurons in both central and peripheral nervous systems (114, 115). Over 50 proteins with neurotrophic properties have been identified in the brain and they are grouped into families according to similarity and properties. The group of “classic neurotrophins” consists of: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) (116). Studies on neurotrophins and their relation to the pathogenesis of AD, focus mainly on NGF and BDNF and neurons in the hippocampus and CBF (99). All of the neurotrophins are produced initially as precursor pro-peptides, after which they are cleaved to produce a smaller mature neurotrophin. The pro-peptides have different properties regarding receptor binding and different biological functions than their mature forms (117).

1.4.1 Neurotrophin receptors in AD

The function of neurotrophins is dependent on their main receptors, a selective tyrosine kinase (Trk) and a pan-neurotrophic p75 neurotrophin ($p75^{NTR}$) receptor. Neurotrophin binding to Trk receptors leads to tyrosine phosphorylation of proteins and promotion of

signal cascades generally associated with neuronal survival, development and maintenance of function in the human central and peripheral nervous systems (118). Mature NGF (matNGF) binds TrkA while BDNF binds TrkB. Both mature and pro-forms of neurotrophins bind p75^{NTR}, although p75^{NTR} is more effectively activated by pro-neurotrophins (119). This generally induces apoptotic signals, though depending on several factors such as co-expression of Trks (120) and ligand binding to the co-receptor sortilin (121, 122). Postmortem studies on AD brains have demonstrated a loss of neurons expressing TrkA and p75^{NTR} (123, 124), and this decline correlates well with cognitive impairment (123). MatNGF binds p75^{NTR} with low affinity (125) while proNGF binds p75^{NTR} with high affinity (119). Activation of pathways through p75^{NTR} can trigger either a pro- or anti-apoptotic signal, depending on the cellular milieu, such as the cell type and the presence or absence of co-receptors (126). MatNGF binds TrkA with high affinity when co-expressed with p75^{NTR} (127), and upon binding, TrkA activates signal cascades that promotes survival of the CBF neuronal phenotype by activating transcription factors that alter gene expression (128).

Figure 4. Schematic representation of the NGF receptor functions. ProNGF induces programmed cell death (apoptosis) via the p75^{NTR} and co-receptor sortilin, while mature NGF induces cell growth and survival via TrkA and p75^{NTR}. Modified from Longo et al 2013 (129).

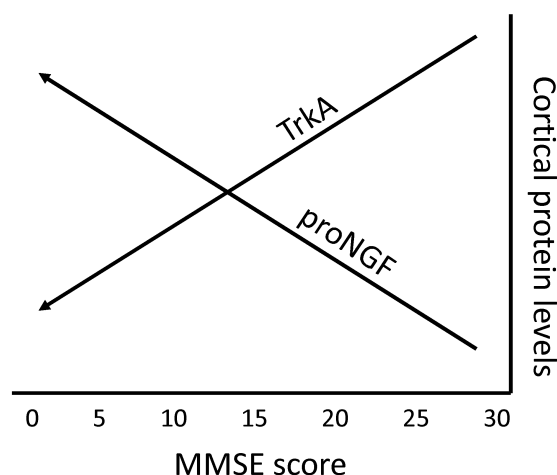


1.4.2 Nerve Growth Factor

Ideas about the biological function of NGF were early on dominated by findings from studies on the survival and differentiation of developing neurons, but it has since been demonstrated that the role of NGF extends beyond the developmental period and beyond the nervous system (130). NGF has a well-established survival-enhancing effect on CBF neurons in animals (131-133), and studies have shown that transgenic adult mice expressing anti-NGF antibodies, display an age-dependent loss of CBF neurons (134, 135). There is furthermore strong evidence supporting the role of NGF in promotion of neurite outgrowth and neuronal differentiation (136, 137).

MatNGF is synthesized in the CBF neurons' target areas in the hippocampus and cerebral cortex (138). These neurons express both $p75^{\text{NTR}}$ and TrkA receptors. TrkA binds matNGF, with the help of $p75^{\text{NTR}}$ and this complex is then transported retrogradely to the basal forebrain where the matNGF/receptor complex mediates signal transduction (97, 139). Numerous studies have demonstrated the importance of retrograde transport of neurotrophin signaling, in both nervous system development and in preventing neuronal degeneration (140). Degeneration of neuronal axons (axonopathy) is thought to precede neuronal cell death, and retrograde transport of neurotrophic factor/receptor complex provides trophic support to the axons, which is thought to halt or prevent axonal degeneration (141). In cortical projections in AD brains, TrkA gene expression (142) and protein levels (123) have been shown to be reduced, while cortical $p75^{\text{NTR}}$ protein levels remain unaltered (143).

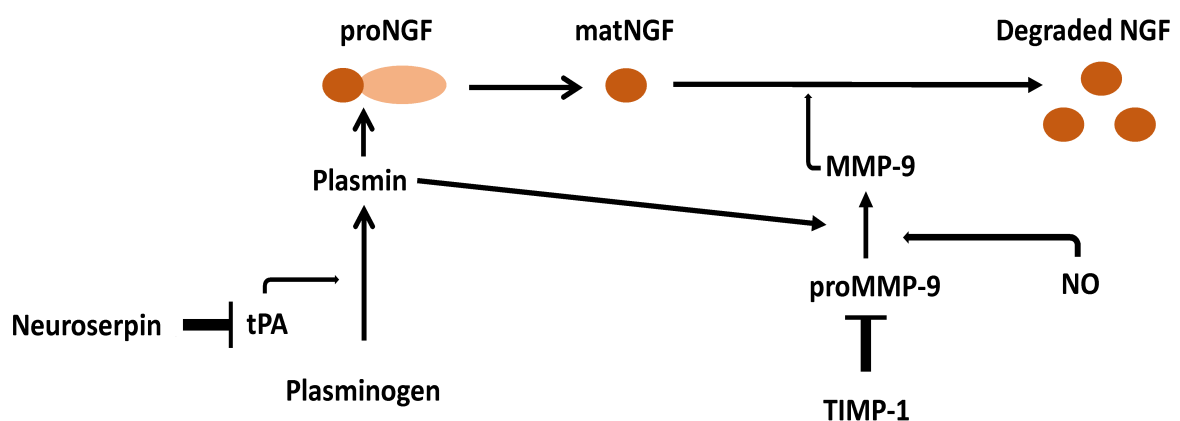
Figure 5. Schematic figure representing findings of decreased cortical levels of TrkA and increased levels of proNGF during the course of AD, correlating with the Mini-mental state examination (MMSE) score. Modified from Counts et al, 2005 (143).



In AD, the ratio of p75^{NTR} to TrkA is suggested to be altered in favor of increased p75^{NTR} mediated activity, which in turn may promote CBF neuron apoptosis. ProNGF has furthermore been shown to be increased twofold in severe AD postmortem cortices, compared to non-demented controls (144), and proNGF is also increased in MCI and mild AD (145), while NGF mRNA has been shown to be unchanged (146). As a result, it has been hypothesized that reduced TrkA and increased levels of proNGF in the AD brain result in a shift towards a proNGF mediated pro-apoptotic signaling, exacerbating a shift from cell survival towards neurodegeneration (143, 147, 148).

Research findings have revealed that proNGF is the main releasable form of NGF following a cerebral cortex stimulation (149) and the conversion of proNGF to matNGF and degradation of matNGF occurs in the extracellular space involving a highly complex protease cascade (150). Considering that studies have demonstrated an imbalance in this maturation pathway, it has been hypothesized that the CBF neurons' trophic status is decided by the levels of the maturation and degradation pathways, rather than protein levels of proNGF (149).

Figure 6. Schematic representation of the maturation and degradation pathways of NGF. Modified from Cuello et al, 2010 (149).



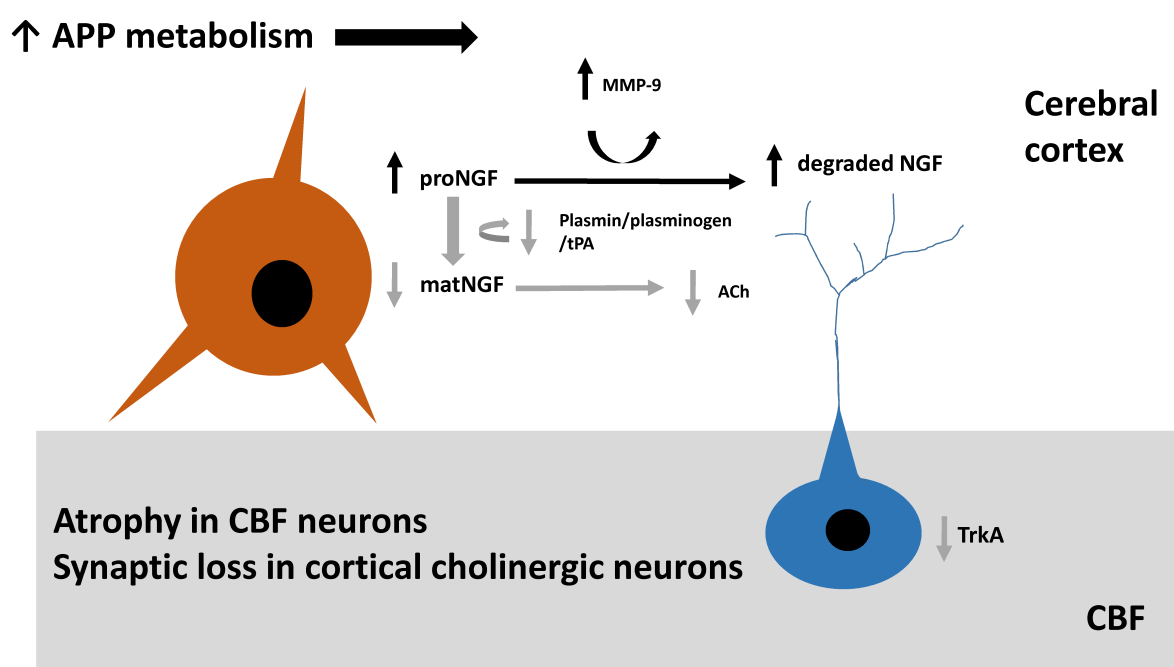
MatNGF is converted from proNGF through activation by plasmin, which is converted from plasminogen through stimulation of tissue plasminogen activator (tPA). Degradation of NGF is mediated by matrix metalloproteinase 9 (MMP-9), which itself has a precursor, proMMP-9, and is matured by way of plasmin and other agents, and regulated by tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) (149). A study on postmortem AD cerebral cortices demonstrated lower levels of plasminogen and higher levels of MMP-9

compared to non-demented controls (151), indicating a failure in the maturation and degradation pathways causing less maturation of NGF and increased degradation, which in turn may lead to a decreased matNGF and consequently CBF neuron atrophy. This assumption is further strengthened by findings from animal studies demonstrating that pharmacological blocking of NGF maturation reduces the levels of matNGF, increases proNGF and consequently leads to cholinergic degeneration and cognitive impairment in the animals (152). Furthermore, by increasing matNGF levels by blocking the NGF degradation pathway, the researchers found an increase in the density of cortical cholinergic boutons (152).

1.4.3 Link to A β pathology

In postmortem AD brains, MMP-9 expression is localized primarily in neurons, amyloid plaques and neurofibrillary tangles (153) and animal studies have demonstrated that A β stimulates the production of several MMP isomers (154), suggesting a coupling to A β and tau pathology in AD.

Figure 7. Schematic representation of the effects on NGF metabolism from a shift towards an amyloidogenic pathway after an induction by injection of A β in a transgenic animal model. Modified from Cuello et al, 2010 (149).



TrkA has been shown to reduce the β -secretase (BACE1) cleavage of APP, in favor of the non-amyloidogenic pathway, while p75^{NTR} increases BACE1 cleavage (155), stimulating the amyloidogenic pathway. Results from animal studies have furthermore demonstrated that increased APP expression in a mouse model of Down's syndrome reduces retrograde transport of NGF, resulting in degeneration of CBF neurons, which on the other hand can be reversed by NGF administration directly to the CBF neuronal bodies (156). It has also been hypothesized that hyperphosphorylated tau may physically hinder the retrograde transport of NGF (157).

1.4.3.1 Previous clinical trials investigating direct delivery of NGF

Results from animal studies demonstrate that the CBF neurons are viable albeit dysfunctional in AD and these cells die late in the disease (133, 158-160), indicating that these degenerating cells may be amenable to therapeutic interventions in early and probably moderate AD (161). It has been proposed that NGF may have a possible disease modifying therapeutic potential in AD for the last 30 years based on the hypothesis that NGF may slow down or stop CBF neuronal loss, thereby halting disease progression. However, NGF is a large molecule which does not pass the blood-brain barrier, posing a challenge with regard to route of administration. Different strategies of direct delivery of NGF have been tested, such as intranasal administration of NGF (162) and a cell-based delivery using stem cells (163). In a previous clinical trial, NGF was administered via infusion into the lateral ventricle of the brain of three AD patients. The patients showed an up-regulation of ACh receptors and increased glucose metabolism, as demonstrated by PET and a normalization of brain electrical activity on electroencephalography (EEG) (164, 165). Unfortunately, the patients experienced adverse events, the most prominent being muscle pain, but also weight loss, making this administration route unfeasible. In clinical studies with NGF administration in diabetes peripheral neuropathy and HIV-associated neuropathy, the patients also experienced adverse events in the form of muscle pain (166, 167).

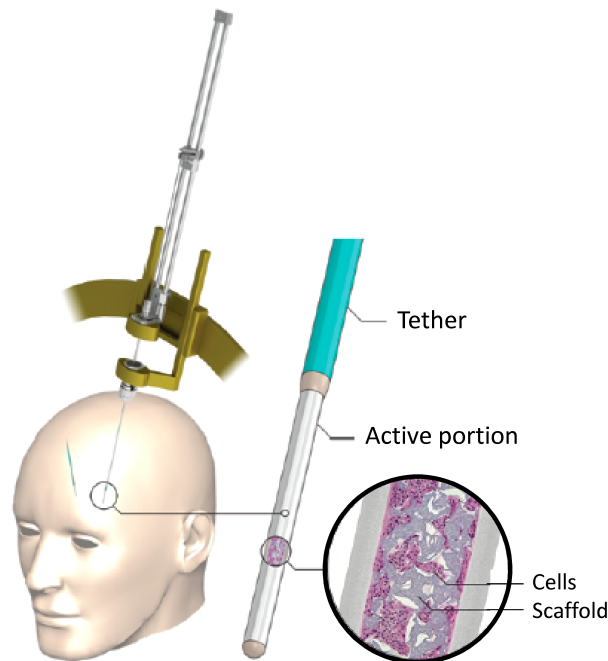
Studies on animals have later shown that pain induced by NGF administration is caused by NGF affecting the neurons in the dorsal root ganglion and hypothalamus, eliciting pain response and weight loss (168). When NGF was infused directly into the rat brain parenchyma (169), and into the basal forebrain in cognitively impaired primates (170), no pain-related side-effects were observed. Further substantiation for the safety of intra-parenchymal administration of NGF was obtained from a study on Parkinson's patients who received direct NGF infusion into the striatum and did not experience increased pain as side-effects (171). It has thus been suggested that in AD patients, NGF should be administered directly to the target neurons in the CBF to avoid side-effects. This is however a challenge with regard to clinical and technical methodologies.

The first study with NGF delivery to the CBF used gene therapy with the patients' own cells, fibroblasts genetically engineered to express and release NGF, then injected stereotactically into the CBF (172). The researchers reported no NGF-related side-effects, and the patients exhibited a possible slowing in cognitive decline and an increased glucose metabolism on FDG-PET. A subsequent phase 1 gene therapy trial in ten AD patients, using a viral vector (AAV2-NGF) with a two-year follow-up also reported safety and tolerability of the NGF transfer (173). A phase 2 multicenter double-blind, sham-surgery controlled trial reported safety and feasibility but failed to demonstrate cognitive improvement (174). A post-mortem report from brain autopsies from ten AD patients from the two phase 1 trials confirmed trophic effects on CBF neurons from the NGF delivery (175). Gene therapy provides an opportunity to deliver a growth factor focally but it may however have hypothetical disadvantages and safety limitations, such as genetic modifications of neuronal cells and the lack of possibility to remove the active substance in case of complications.

1.4.3.2 Encapsulated cell biodelivery of NGF

Another alternative is to utilize encapsulated cells that can be implanted by way of stereotactic neurosurgery and removed if required. In collaboration with the biotech company NsGene Inc., our research group developed a biodelivery device containing *ex vivo* genetically modified, encapsulated NGF releasing cells. The capsule provides immunoisolation while oxygen, nutrients and the therapeutic product flows freely over the capsule wall. In a phase I clinical trial (presented in **papers II – IV**), the biodelivery device (NGF-ECB implant) was implanted stereotactically into the CBF of six AD patients during a 12-month follow-up. No NGF attributable side effects were reported and the results demonstrated safety and feasibility of NGF delivery by NGF-ECB implantation (176, 177). This approach has the advantage compared to gene therapy that delivery of the therapeutic agent can be stopped and the cells removed from the brain, without the patient's genes being altered.

Figure 8. Schematic representation of the NGF-ECB device. Published with the permission of Lars U. Wahlberg.



1.4.4 Brain-derived neurotrophic factor

Changes in the levels of BDNF have been linked to the pathophysiology of a number of disorders in the central nervous system such as AD, Huntington's disease, depression and schizophrenia (99). BDNF is expressed and distributed widely throughout the brain, though especially (178, 179) in neurons in the cerebral cortex and hippocampus and it has been shown to be important for the function and survival of both dopaminergic and cholinergic neurons (180, 181). Mature BDNF regulates differentiation and plasticity of these neurons by activation of its high-affinity receptor TrkB and low-affinity receptor $p75^{\text{NTR}}$ (182).

Long-term potentiation (LTP) has been described as a "persistent increase in synaptic strength induced by brief high-frequency electrical stimulation of afferent fibres or coincident activation of pre- and postsynaptic neurons" (183) and is widely accepted as a primary hypothesis for the mechanism of learning on a cellular level. The role of BDNF in LTP has been established in animal experiments, which have demonstrated that LTP in hippocampal neurons is enhanced by overexpression of TrkB (184), while reduced BDNF-TrkB signaling leads to memory impairment (183). Some studies have reported lower levels of TrkB in AD brains (185), but the literature is inconsistent regarding this (186). Declining cortical levels of BDNF in AD correlate to increased Braak staging, a staging

based on the distribution of tau pathology in AD (186) and one study has also reported declined levels of BDNF mRNA in non-AD tauopathies (187). However, truncated TrkB.T1 receptor, which inhibits both TrkB and p75^{NTR} receptors (188, 189), and inhibits LTP in animal models (189), has been found to be increased in cerebral cortices from AD patients (190). Moreover, proBDNF signaling through binding p75^{NTR} promotes long-term neuronal depression (191). Additionally, BDNF may have a protective effect against A β toxicity both *in vivo* and *in vitro* (192, 193) and prevent A β induced LTP impairment in brain slices from hippocampal (194) and entorhinal cortices (195). As is the case for NGF, BDNF's pharmacokinetic properties are ill-suited for systemic administration due to poor blood-brain barrier penetration and short half-life in plasma.

1.4.5 Levels of NGF, BDNF and their respective pro-forms in CSF

The presence of mature and pro-forms of BDNF and NGF in CSF is not yet well characterized. Several studies have however investigated levels of neurotrophins in postmortem cortical and hippocampal slices and found elevated levels of proNGF in AD and MCI compared to subjects with no cognitive impairment (145, 196) and decreased levels of proBDNF in postmortem brains of AD and MCI patients (197, 198). A recent paper reported increased levels of proNGF in ventricular CSF from deceased AD and MCI patients compared to non-demented controls, but the researchers could only detect matNGF in a few CSF samples (199). Other reports have found increased matNGF in CSF from AD patients (200, 201). Previous studies have reported either decreased levels of BDNF in CSF from AD patients compared to non-demented controls or other types of dementia (202, 203), or found no significant difference (204), although none of these studies differentiated between mature and proBDNF.

A few studies have investigated the association between levels of BDNF and risk of progression of cognitive decline and development of AD dementia. Weinstein et al. reported, using ELISA, lower levels of serum BDNF in MCI patients who at follow-up had progressed to AD dementia in a cohort from the Framingham Heart Study (205). Other reports described higher levels of serum/plasma BDNF in MCI and early AD patients compared to healthy controls (206, 207), suggesting a compensatory mechanism in MCI in response to a developing AD pathology. One study reported lower levels of BDNF in CSF in AD patients in comparison to MCI and non-demented controls, and furthermore that lower BDNF levels seemed to be related to higher risk for conversion from MCI to AD (208).

In the light of the important roles that NGF and BDNF play in cognition, it will be valuable to be able to investigate the levels of both their mature and pro-forms to establish a better understanding of the disease mechanism in AD and furthermore to develop strategies for better identification of individuals at increased risk of progression to AD.

2 AIMS

NGF delivery to the cholinergic basal forebrain has been suggested as a potential disease-modifying treatment in AD, and different delivery methods have been investigated for the last three decades.

The main aim of this thesis is to explore the potential of encapsulated cell biodelivery of NGF, targeted to the basal forebrain, with regard to safety, tolerability and a possible disease-modifying effect, in a total of ten AD patients.

Specifically, the aims include investigating:

- levels of the neurotrophins NGF and BDNF and their pro-peptides in CSF from subjects with AD as compared to levels in subjects with mild cognitive impairment and subjective cognitive impairment (**paper I**),
- whether changes in CSF cholinergic markers from AD patients receiving NGF delivery for twelve months, have a potential for monitoring changes in cholinergic activity of the brain induced by the NGF delivery (**paper II**),
- potential effects of NGF delivery on quantitative EEG parameters, and if such changes correlate with cognitive outcome or CSF cholinergic markers (**paper III**),
- possible changes in brain atrophy on MRI during NGF delivery in comparison to a control group, and correlative pattern with cognition and CSF biomarkers (**paper IV**),
- safety and tolerability of implantation with a second-generation NGF-ECB device in four AD patients, as well as effects on secondary outcome measures such as cognition and biomarkers (**paper V**).

3 SUBJECTS AND METHODS

In this thesis, several methods are used, spanning from cognitive tests, imaging and neurophysiological investigations, to analyses of levels of CSF biomarkers. Here, a brief account of the methods is given. Please see the respective papers for a more detailed description.

3.1 SUBJECTS

3.1.1 Participants in a two dose-step clinical trial with NGF-ECB (papers II-V)

Between 2007 and 2008, our research group conducted a 12-month open-label, single center phase I trial of a **first-in-man** NGF-EC biodelivery to the CBF in six AD patients, in two dose cohorts. In 2011-2012, a dose-escalation step was performed, adding a third cohort of four AD patients to the trial cohort.

The ten participants were enrolled to both steps of the trial from the Memory Clinic at Karolinska University Hospital, Huddinge. We used the same inclusion and exclusion criteria for all three cohorts, which were: a) probable diagnosis of mild to moderate AD according to the NINCDS-ADRDA criteria (42), b) age between 50 and 80 years, c) Mini-mental state examination (MMSE) (209) score between 15 and 24, d) living at home with a caregiver, e) stable ChEI treatment for at least three months prior to enrollment. The exclusion criteria were: a) medical and/or psychiatric comorbidities which would make it difficult for the patients to undergo the surgical procedure including general anesthesia, and complete the trial, b) ongoing treatment with antipsychotic drugs and c) smoking, not to interfere with nicotinic receptor binding on PET. We screened approximately 100 patient charts to identify candidates who fulfilled the inclusion criteria. Patients were thereafter contacted with a short letter with information on the trial, followed up with a phone call, after which patient information was sent home to the patient and caregiver. Next, the patients came for a visit at the Memory Clinic to discuss enrollment. If the patient and caregiver gave an informed written consent, the screening visit was initiated. Brain MRI was performed on all patients at enrollment to exclude significant neurological conditions that would contradict surgery. For cohort III, six patients performed the screening visit, five were enrolled and one patient was excluded after MRI due to signs of an old brain hemorrhage.

Of note is that the AD diagnosis was confirmed neuropathologically by small cortical biopsies obtained at NGF-ECB implantation, exhibiting plaques and tangles in biopsies from nine out of ten patients (176, 210), whereas one biopsy showed only fat tissue (176).

3.1.2 Participants in a study on CSF levels of neurotrophins (paper I)

The subjects were individuals that had been referred to the Memory Clinic at Karolinska University Hospital Huddinge, Stockholm between 2007 and 2009, due to memory complaints, and received a diagnosis of AD, MCI or SCI. All subjects underwent a work-up according to a clinical routine at the Memory Clinic and following medical history, history from a proxy and clinical examination including neurological and psychiatric assessments, cognitive screening was performed using MMSE. Additionally, the subjects were assessed by brain imaging (MRI or computerized tomography (CT)), blood tests and biomarkers in CSF. A majority of the subjects were assessed with a neuropsychological test battery. AD diagnosis was established according to the ICD-10 criteria (40), and MCI according to the Winblad criteria (49) (see *section 1.1.4.1*). For establishing a diagnosis of SCI, the definition of SCI as a presence of cognitive complaints in the absence of pathological neuropsychological testing was used (211, 212). All subjects signed an informed consent prior to lumbar puncture (LP) and approved to donate CSF to a biobank.

The inclusion criteria were: a) LP performed as a part of the work-up, b) diagnosis of AD, MCI or SCI. Exclusion criteria were: a) somatic or psychiatric disease significantly affecting cognition, b) non-AD dementia and c) first degree relative with AD among the subjects in the SCI group. CSF from a total of 96 patients were collected, 22 AD patients, 35 MCI, and 39 SCI subjects.

3.2 METHODS

3.2.1 Analyses of neurotrophins and their pro-forms in CSF (paper I)

After LP, the CSF samples were stored in -80° C until analysis. The CSF levels of A β ₄₂, t-tau and p-tau were assessed by commercially available sandwich ELISAs according to standardized protocols.

We used immunoblotting by western blot and electrophoresis on an SDS-PAGE gel in order to investigate the presence of mature and pro-forms of NGF and BDNF in CSF. The levels of different forms of NGF and BDNF were expected to be low, and for that reason we lyophilized the CSF samples by vacuum centrifugation by which the samples were concentrated. Determination of total protein was done in each sample prior to lyophilizing and concentrating the samples and the lowest protein concentration in each sample was set to 1.5 μ g/ml, thus enabling us to analyze the same amount of protein from each subject by electrophoresis and immunoblotting. Only immunoreactive bands that could be proven to be specific were analyzed. The intensity of the signals from specific bands were normalized to the intensity of an internal control, present in all runs. This normalized data

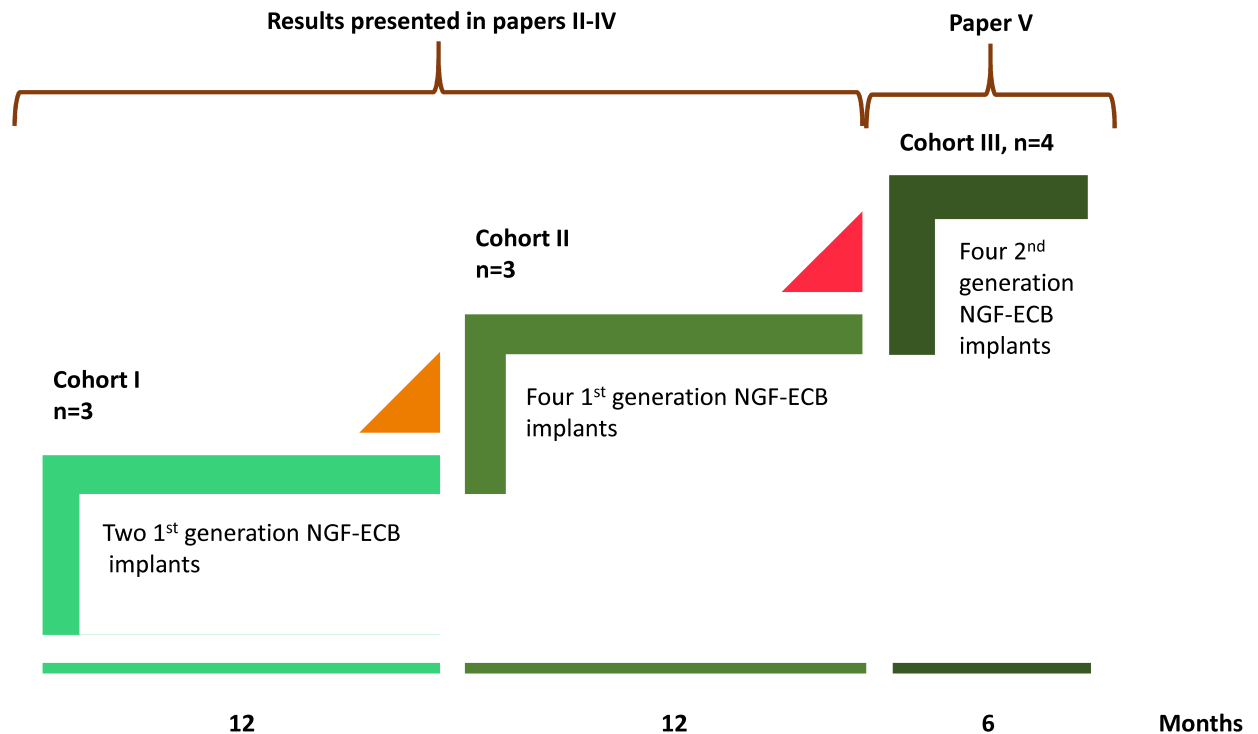
was thereafter multiplied with the factor by which the sample was diluted to attain the concentration 1.5 µg/ml. The resulting data, expressed in arbitrary units (a.u.), reflects the neurotrophin levels present in CSF in the way that relative comparisons between subjects are possible. The differences between the diagnostic groups were thus analyzed, as well as correlations with biomarkers and MMSE score. To investigate if an association between progression to dementia and levels of neurotrophins existed, the subjects' medical charts were inspected to explore if the subjects had been followed up, and if so if they had converted from MCI to AD. Subjects who had converted were assigned as *converters* and those who remained cognitively stable were assigned as *non-converters*, according to the clinical diagnosis at the time of follow-up.

A ratio of t-tau/Aβ₄₂>0.52 has been shown have a high sensitivity and specificity (66) for differentiating AD from healthy individuals, and such a biomarker profile can be regarded as pathological (66). To investigate if an association between biomarker profile and neurotrophin levels existed, we performed correlative analyses between CSF neurotrophin levels and the biomarker status (*pathological* or *non-pathological* biomarker profile).

3.2.2 Encapsulated cell biodelivery of NGF

To the first arm of this phase I NGF-ECB trial, as previously reported (176, 177), six AD patients were enrolled in two dose cohorts. *Cohort I* (three patients) received two NGF-ECB implants, stereotactically implanted bilaterally in the nucleus basalis of Meynert (Ch4) while *cohort II* (three patients) received double bilateral NGF-ECB implants (four implants), implanted in Ch4 and in the vertical limb of the diagonal band of Broca (Ch2). The duration of NGF delivery in *cohorts I & II* was twelve months. *Cohort III* (four patients) received double bilateral second-generation NGF-ECB implants in Ch2 and Ch4 during a trial period of six months.

Figure 9. Schematic representation of the dose-escalation regime of the study. Adapted from Eyjolfsdottir et al, 2016 (210) and reprinted with permission from BioMed Central.



3.2.2.1 First generation NGF-ECB implant (NsG0202), cohorts I & II (papers II-IV)

The implant consisted of human retinal pigment epithelial cells derived from a commercially available cell line (ARPE-19), transfected and modified with the human NGF gene, expressing NGF but no proNGF (213), housed in the active part of the implant, behind a semipermeable membrane (see figure 8). The active part is attached to a 1 mm wide tether (see figure 8).

3.2.2.2 Second generation NGF-ECB implant (NsG0202.1), cohort III (paper V)

This implant also consisted of human retinal pigment epithelial cells derived from the ARPE-19 cell line, transfected and modified with the human NGF gene, as described above and reported by Fjord-Larsen et al, 2012 (214). The cells were now co-transfected with separate plasmids coding for human NGF and a Sleeping Beauty transposase (215). The polyvinyl alcohol sponge matrix used in the first-generation implant was replaced by a polyester terephthalate yarn matrix as supportive scaffold for the cells, allowing improved cell adherence and survival.

Both implants NsG0202 and NsG0202.1 were tested for safety and toxicology in animals and produced under Good Manufacturing Practice (GMP).

3.2.2.3 Neurosurgical procedures

The implantation and implant retrieval (explantation) procedures were performed by a neurosurgical team under general anesthesia. For *cohort I*, the three patients were implanted stereotactically with bilateral single implants with the active part targeting Ch4. The tether was thereafter cut to length and secured at the burr hole with a titanium plate (177).

Three months later, safety data were assessed by an independent review board, and based on the assessment, three patients (*cohort II*), were approved for implantation of bilateral double implants to Ch2 and Ch4. In *cohort III (paper V)*, the procedures were performed in the same manner as described for *cohorts I & II* to the same anatomical targets as described for the first *two* dose cohorts.

Immediately post operatively, cranial CT scans were performed on all patients for safety assessment and documentation of implant positions. At the end of the trial period for *cohort III* at six months and for *cohorts I & II* at twelve months, all ten patients underwent surgical retrieval of the implants under general anesthesia according to a protocol described by Eriksdotter-Jönhagen et al, 2012 and Wahlberg et al, 2012 (176, 177).

3.2.2.4 Outcome measures

Primary outcome measures for all three dose cohorts of the NGF-ECB clinical trial were safety and tolerability, assessed by clinical follow-up with regard to adverse events (AEs). At baseline, all patients were examined thoroughly by a physician and investigated with cranial MRI and laboratory tests. At 3 and 6 month follow-ups, this was repeated in *cohort III*, and furthermore at 12 months for *cohorts I & II*. Clinical assessments were intensified close to surgery with daily assessments of safety and tolerability parameters. Patients were admitted, first to a neurosurgery ward, followed by a geriatric ward for a mean stay of 5 days. After discharge, the patients were monitored closely with outpatients visits one, two and four weeks after surgery. A similar intensified monitoring was performed upon implant retrieval, with a hospital stay for a mean duration of two days. The study physician (HE) was on standby during the whole duration of the trial and could always be reached by phone.

Secondary outcome measures included: a) cognitive status, assessed with cognitive screening tests (MMSE and Alzheimer's Disease Assessment Scale-Cognitive Subscale

(ADAS-Cog) (216)) and neuropsychological test battery, b) quantitative EEG (**paper III**), c) CSF analyses for AD biomarkers ($A\beta_{42}$, t-tau and p-tau, measured with xMAP technology (217), neurofilament light protein (NFL) and glial fibrillary acidic protein (GFAP), analysed with ELISA assays (218, 219) and the cholinergic markers ChAT and AChE (**paper II**), d) MRI for assessment of atrophy (**paper IV**) and e) PET investigation for assessment of glucose utilization (FDG-PET) and nicotinic receptor binding at baseline, 3 and 6 months (*cohort III*) or 12 months (*cohorts I & II*), as a putative surrogate of the number of nicotinic ACh receptors. The ^{11}C -nicotinic binding sites were assessed using a nicotine tracer (220). Thereafter, we calculated a flow-compensated parameter, k_2^* for nicotine divided by cerebral blood flow (221, 222), but since k_2^* has an inverse relationship to number of nicotinic binding sites, we transformed the values by calculating $1-k_2^*$.

3.2.3 Cholinergic markers in CSF fluid following NGF delivery (paper II)

CSF levels of the cholinergic markers ChAT and AChE were measured from the six AD patients in *cohorts I* and *II*, who were implanted with first generation NGF-ECB implants. CSF ChAT activity was measured by a newly developed colorimetric assay (223) and AChE activity by modified Ellman's colorimetric assay as described by Darreh-Shori et al, 2008 (224). Annual cognitive decline in untreated AD patients has been reported to be 3-5 points/year and 2.5 points/year in ChEI treated patients, assessed one year after ChEI therapy start (225, 226). In this study, three patients showed a ≤ 2 points annual decline in MMSE score after 12 months of NGF delivery and were assigned as *responders*, while three patients showed > 2 points annual decline and were assigned as *non-responders*.

For comparison with an AD group without NGF delivery, but with ChEI treatment for a similar duration as the NGF patients, we included CSF samples from 17 AD patients, from whom CSF had been collected at 12 and 24 months of ChEI treatment (227). Inclusion criteria were availability of CSF samples and MMSE scores both after 12 and 24 months of stable ChEI therapy. The same criteria for assigning to *responder* or *non-responder* groups according to annual MMSE decline was applied.

We analyzed changes from baseline in the cholinergic markers at 3 and 12 month of NGF delivery and performed correlative analyses using Spearman rank non-parametric correlation between the cholinergic markers, cognitive performance, glucose utilization and nicotinic binding on PET.

3.2.4 Changes in EEG activity following NGF delivery (paper III)

In the six AD patients in dose *cohorts I & II*, standard EEGs were recorded according to the international 10/20 system in a resting awake state, with vigilance control. After digitizing

at a sampling rate of 256 Hz, absolute power for 19 standard electrode locations and Global Field Power (GFP), corresponding to the generalized EEG amplitude, was calculated using the Brain Vision Analyzer software. In addition, the absolute power and GFP were averaged in six frequency bands: delta (1-3.5 Hz), theta (4-7.5 Hz), alpha (8-11.5 Hz), beta 1 (12-15.5 Hz), beta 2 (16-19.5 Hz) and beta 3 (20-23.5 Hz). Values were logarithmically transformed for normalization of data distribution.

Advanced statistical analyses of differences of the amplitude and spatial distribution of the EEG absolute power across the whole spectra (0-70 Hz) (not averaged across the frequency bands) were performed by the statistical software Randomized Graphical User Interface (Ragu) (228), for topographical analyses of covariance (TANCOVAs) (229). Ragu tests whether a significant amount of topographic variance can be accounted for by the linear contribution of an external predictor that may vary by different conditions. In this paper, we used MMSE and ChAT changes (post treatment – pretreatment) as external predictors and the recording occasions at baseline, 3 and 12 months of NGF delivery were assigned as conditions.

3.2.5 Atrophy parameters on MRI following NGF delivery (paper IV)

The six AD patients from *cohorts I & II* were scanned on a 1.5T MR scanner (details presented in **paper IV**). Images were processed according to a specific protocol to acquire reliable volume estimates. Visual quality control was performed on the output data.

An index of brain atrophy was calculated using the following formula: (total gray matter volume + total white matter volume) / total CSF volume. The index represents brain volume (BV) in relation to total volume of CSF at a given follow-up point. Lower values of BV/CSF index represent greater brain atrophy. Brain volume has a tendency to decline in both normal aging as well as neurodegenerative disease while CSF increases. We furthermore analyzed hippocampal volume for each patient and normalized to each subjects' intracranial volume (230).

To establish a reference group for comparisons of brain atrophy to an age-matched group of AD patients without NGF delivery, a dataset from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (231) was obtained from 36 AD patients with a 12-month MRI follow-up and who also were scanned on a 1.5T MRI system (232). Diagnostic criteria for the ADNI cohort were as follows: a) diagnosis of probable AD according to the NINCDS-ADRDA criteria, b) Clinical Dementia Rating scale (CDR) score between 0.5-1 (233), c) MMSE scores 20-26 and d) age 55-90 years. Exclusion criteria were: a) significant neurologic disease other than AD and b) concomitant medication with psychoactive medication other than ChEIs and/or stable doses of baseline medication listed in the ADNI procedures manual.

We assigned the enrolled NGF patients to *responders* or *non-responders* groups according

to their annual change in MMSE score, according to the method described in section 3.2.3 and MMSE results from ADNI reference group were treated the same way.

3.3 ETHICAL CONSIDERATIONS

All studies presented in this thesis are conducted according to the Helsinki Declaration and were approved by the Regional Human Ethics Committee of Stockholm (detailed below) and approval was also acquired from the Swedish Medical Products Agency for studies presented in **papers II-V**.

All patients involved in this thesis granted their consent, and consent was furthermore obtained from caregivers of the ten AD patients in *cohorts I-III* of the NGF-ECB trial.

Ethical permissions:

Paper I: 2011/680-31/1,

Papers II, III and IV: 2007/986-31/3,

Paper V: 2011/1048-31, and subsequent amendment 2011/1874-32.

4 RESULTS AND DISCUSSION

4.1 ANALYSES OF NEUROTROPHINS AND THEIR PRO-FORMS IN CSF (PAPER I)

CSF samples from a total of 96 patients were analyzed for NGF, BDNF and their respective pro-forms. Demographic data on the subjects are summarized in *Table 1*.

Table 1. Demographic data on included subjects. * indicates a statistically significant difference as compared to AD ($p < 0.05$), # indicates significance as compared to MCI ($p < 0.05$).

	All subjects	SCI	MCI	AD
N	96	39	35	22
(women/men)	(61/35)	(24/15)	(23/12)	(14/8)
	median (range)	median (range)	median (range)	median (range)
Age at LP	64 (42-86)	61# (43-86)	69 (42-86)	66 (55-84)
t-tau (ng/L)	266 (75-1470)	207*# (75-728)	276* (79-510)	549 (209-1470)
A β 42 (ng/L)	635 (300-1334)	980*# (300-1216)	586* (350-1334)	493 (332-840)
p-tau (ng/L)	59 (16-182)	49* (16-110)	56* (16-95)	83 (45-182)
t-tau/A β ₄₂	0.36 (0.1-3.55)	0.21*# (0.1-1.87)	0.45* (0.12-1.2)	1.16 (0.36-3.55)
MMSE score	28 (15-30)	29*# (23-30)	28* (23-30)	23 (15-26)

4.1.1 Immunoblotting experiments and antibody specificity

By applying a western blot method on CSF, we discovered protein bands of specific immunoreactivity for BDNF at 12-14 and 24 kDa and we concluded that mature BDNF (matBDNF) at 12-14 kDa could be detected and a form of proBDNF at 24 kDa in human CSF samples. The staining for NGF produced similar results with a band identified as matNGF at 12-14 kDa and a band identified as proNGF at 24 kDa. These results are in accordance with previous studies showing forms of BDNF and NGF at these molecular weights (234-238).

We could not consistently produce positive stainings for the mature neurotrophins in CSF. We believe that this may be to technical limitations and that these forms indeed are present in human CSF, although it has been recognized that proNGF is the predominant form of NGF in brain samples (144). Also, it was recently reported that matNGF was variably detectable and could not be reliably quantified in CSF (239). As for BDNF, no study

has thus far, to our knowledge, differentiated between the mature and pro-form when reporting levels in CSF.

4.1.2 Levels of pro-neurotrophins between diagnostic groups

Our results show significantly lower levels of proNGF in CSF samples from subjects with MCI compared with AD ($p < 0.05$) but compared to SCI, the difference was not significant ($p = 0.056$). No significant differences in the levels of proBDNF were found between the diagnostic groups. Previous reports describe higher levels of NGF in CSF from AD patients (201, 204), but most studies thus far have, however, used ELISA for neurotrophin detection. We argue that results based on ELISA should be interpreted with caution due to difficulties in separating pro and mature forms of neurotrophins, since proteins containing the same epitope, such as in the case of mature and pro-forms of NGF and BDNF, will be detected together in the same ELISA assay. A recent report did however demonstrate significantly higher levels of proNGF in postmortem CSF from AD and MCI subjects, compared to subjects with no cognitive impairment (239), using western blot and ELISA. We believe that future studies on CSF samples from larger cohorts of AD and MCI subjects may help clarify if there is a difference in the levels of proNGF between these groups as indicated by our data.

The choice of an SCI group as non-demented controls can be criticized considering that the group consists of individuals who were referred to a memory clinic due to complaints of memory loss. They were, however, investigated extensively, including a neuropsychological evaluation in most cases, CSF biomarkers and imaging and no objective memory impairment could be verified. Accordingly, we refer to them as cognitively healthy at the time of inclusion to the study (time of LP), although several reports have demonstrated that SCI may be associated with a significantly higher risk of developing dementia (240).

4.1.3 Neurotrophins and relation to a pathological biomarker profile and a progress of cognitive impairment

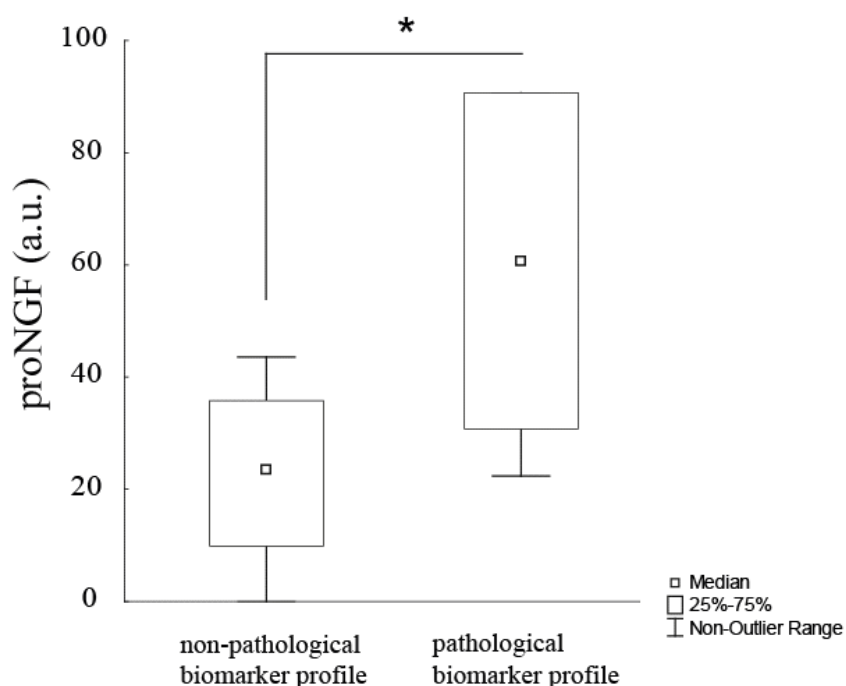
In the MCI group, 80% had been followed up 7-9 years after the first LP and of those, 39% had converted to AD dementia and one patient to Lewy body dementia. In the SCI group, 23% were followed up and only one subject converted to MCI. Due to a low follow-up rate in the SCI group, only data from the MCI subjects were analyzed. The MCI subjects who converted to AD were slightly older than the *non-converters*, but the difference was not significant nor did the groups differ significantly with regard to pathological biomarker profile ($t\text{-tau}/A\beta_{42} > 0.52$). Our data showed that MCI subjects who converted to AD

(*converters*) showed significantly lower levels of proBDNF in CSF ($p<0.05$) compared to those subjects who remained cognitively stable (*non-converters*). There was no significant difference in CSF levels of proNGF in relation to conversion to AD.

We suggest that in MCI, there may exist a deficiency in production or metabolism of BDNF, reflecting a pathological process in the brain. These findings are in accordance with a report from Peng et al describing lower levels of both pro- and matBDNF in the brain of pre-clinical AD patients compared to non-demented controls (197), suggesting that lower levels of proBDNF in CSF reflect the situation in the brain. Further studies are needed to investigate if lower levels of proNGF and proBDNF are due to a decreased production, altered metabolism or both.

When analyzing pro-neurotrophins and the association to a pathological biomarker profile, we found that MCI subjects with such a biomarker profile showed higher levels of CSF proNGF (*figure 10*), but no significant difference was found with regard to proBDNF.

Figure 10. CSF levels of proNGF in MCI subjects with or without a pathological biomarker profile ($t\text{-tau}/A\beta>0.52$). ProNGF levels were significantly higher in subjects with a pathological biomarker profile than with a normal (non-pathological) biomarker profile. Y-axis represents percentage of internal control. Statistical significance is indicated by * ($p<0.05$).



Metabolisms of pro- and mature forms of NGF and BDNF are mediated by complex enzyme cascades, involving e.g. plasmin (241), and metalloproteases (MMPs) (242), which are controlled by activators and inhibitors (see *Figure 6*). A neurodegenerative process that affects these enzymatic pathways could well be reflected in changes in levels of NGF and BDNF. Our research group has previously reported higher levels of plasminogen, neuroserpin and tissue inhibitor of metalloproteases-1 (TIMP-1) in CSF from subjects with MCI (243). Changes in these enzyme cascades in AD may thus help explain our results and give further strength to a hypothesis that neurotrophin dysfunction and imbalance in MCI may be associated with a progression to AD.

4.2 ENCAPSULATED CELL BIODELIVERY OF NGF, COHORTS I AND II (PAPERS II-IV)

Table 2. Demographic data on enrolled patients in *cohorts I* and *II* of the NGF-ECB trial, at baseline, 3 and 12 months.

	Baseline median (range)	3 months median (range)	12 months median (range)
Gender, n (M/F)	4 M/2 F		
Age (years)	62 (55-73)		
Memory problems (years)	4 (1-6)		
Time from AD diagnosis (years)	1.5 (1-3)		
Duration of ChEI treatment (months)	12 (8-26)		
MMSE score	23 (19-24)	22 (18-28)	18 (14-27)
ADAS-Cog score	27 (22-34)	25 (22-32)	39 (20-53)
CDR score (sum of boxes)	0.75 (0.5-1)	1 (0.5-1)	1 (0.5-3)
IADL score	14 (9-21)	17 (10-20)	20 (13-30)
CSF Aβ_{42} (ng/L)	138 (117-213)	130 (92-198)	145 (82-209)
CSF p-tau (ng/L)	39 (39-77)	34 (22-81)	40 (20-46)
CSF t-tau (ng/L)	153 (75-180)	114 (65-199)	114 (60-191)
CSF NFL (ng/L)	158 (125-360)	845 (550-3120)	260 (125-420)
CSF GFAP (ng/L)	785 (710-930)	600 (570-1180)	850 (800-1280)
CSF ChAT activity (nmol/min/mL)	2.6 (2.0-3.7)	2.7 (2.3-3.1)	3.01 (2.05-4.35)
CSF AChE activity (nmol/min/mL)	14.7 (8.6-18.4)	13.8 (10.6-23.3)	18.6 (10.1-28.6)

4.3 CHANGES IN CSF CHOLINERGIC MARKERS FOLLOWING NGF DELIVERY (PAPER II)

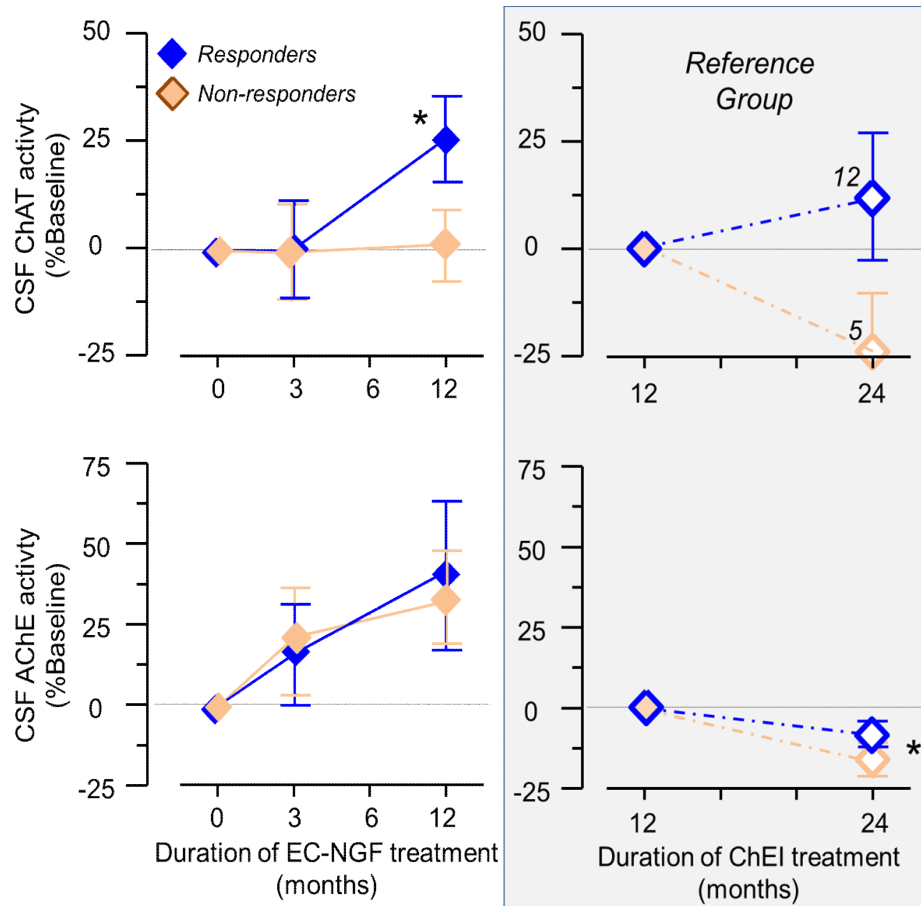
4.3.1 ChAT activity in CSF increased during NGF delivery in *responders*

At 12 months of NGF delivery, the *responder* group (**defined as annual decline in MMSE \leq 2 points**) showed a significant increase in ChAT activity compared to the *non-responder* group ($p < 0.05$) (**defined as annual decline in MMSE > 2 points**) ($p < 0.05$), but we found no significant difference in CSF ChAT activity over 12 months in a reference group of 17 long-term stably ChEI treated AD patients, and neither did we find changes when we divided the reference group to *responders* and *non-responders* according to the same criteria. The presence of extracellular ChAT was recently discovered (223), and this is the first report publishing data on ChAT activity in CSF in response to a long-term stable ChEI treatment. We argue that the observed increase in ChAT activity during NGF delivery cannot be explained by the concomitant ChEI treatment alone, as these changes were not observed in the ChEI treated reference group. On the contrary, ChAT activity amongst the non-responders in the reference group declined during the 12-month period. Furthermore, ChAT activity has been suggested to increase both in vivo and in vitro as a response to NGF administration in rats and *in vitro* (244, 245), giving further support to our findings. The patients' cognitive performance on MMSE was followed up for 15 months and ADAS-Cog for 7 months after removal of the NGF-ECB implants and MMSE assessments were also available for an average of 20 months previous to the trial baseline. We found that the *responders* showed no significant decline in MMSE performance, compared with own score at baseline, and compared to *non-responders* for 15 months post NGF delivery and the same pattern was observed for 3 months post NGF delivery for ADAS-Cog. Thus, we found indications for a cognitive stabilization by NGF delivery that was enduring in the *responders group* for at least 3 months post NGF.

4.3.2 AChE activity in CSF increased in the whole group at 12 months of NGF delivery

The activity of AChE in CSF was increased at 12 months of NGF delivery in the total NGF group, ($p < 0.05$), compared to baseline, but we found no statistically significant difference between the *responder* and *non-responder* groups. The reference group showed a decrease in AChE activity over a 12 month period, most pronounced in the *non-responders* in the reference group ($p < 0.05$).

Figure 11. Changes from baseline in ChAT and AChE activities in CSF at 12 months of NGF delivery. Adapted from Karami, Eyjolfsson et al, 2015 and reprinted with permission from Elsevier (227). * $p < 0.05$: difference from baseline.



Studies demonstrate that following ChEI treatment in AD patients, AChE protein levels and activity in CSF show an increase (224, 246), and that increased enzyme activity reflects enzyme inhibition (247). The increase in CSF AChE activity observed in this study is though unlikely to be explained by ChEI treatment, as the NGF cohort had been treated for at least 8 months before NGF-ECB implantation. Moreover, the reference group did not exhibit the same increase which gives further support to our proposition that the increase in CSF cholinergic markers is likely to be a consequence of stimulation of the cholinergic basal forebrain induced by NGF delivery.

4.3.3 Increase over time in CSF cholinergic markers correlated with cognition, brain glucose utilization and nicotine receptor binding

We found a significant positive correlation between ChAT activity in CSF and performance on MMSE, both for absolute values ($r=0.89$, $p = 0.014$) and percent change from baseline ($r = 0.92$, $p<0.005$), at 12 months of NGF delivery, but not at 3 months. Similar correlative patterns were found when we examined the association between change from baseline in CSF ChAT at 12 months and performance on ADAS-Cog ($r = -0.91$, $p = 0.0093$).

CSF ChAT activity also correlated with *in vivo* brain glucose utilization measured with FDG-PET after 12 months of NGF delivery in the average cortical regions ($r = 0.83$, $p = 0.037$). AChE activity correlated with glucose brain utilization in overall brain regions ($r = 0.82$, $p = 0.043$). ChAT and AChE activities in CSF also correlated with ^{11}C nicotine binding, measured with PET, though particularly in the left hemisphere. Furthermore, changes in levels of ^{11}C -nicotine binding correlated with changes in cognitive performance, measured with MMSE and ADAS-Cog, during a 12 month NGF delivery ($r = 0.82$, $p = 0.048$ and $r = -0.89$, $p = 0.013$, respectively).

The cholinergic system exhibits deficiencies early on in AD, substantiated with reduced levels of ChAT in the brain and a decrease in nicotinic ACh receptors. A disease-modifying treatment to AD therefore has to be able to stop or even reverse the cholinergic deficit. It is intriguing to speculate that the findings from our NGF delivery trial suggest disease-modifying effects **for the first time**.

4.4 EFFECTS ON QUANTITATIVE EEG PARAMETERS FOLLOWING NGF DELIVERY (PAPER III)

Since there is a slowing of the background rhythmic activity in EEG in AD, with reduction of the fast alpha power and increase in the slow theta power, we hypothesized that following the NGF delivery, an increase in alpha and decrease in theta power would occur. Also, in our previous paper on NGF delivery (165) with intracerebroventricular infusion of NGF, positive effects on EEG activity were found. Thus, it was important to study the effects on EEG also in this NGF delivery trial.

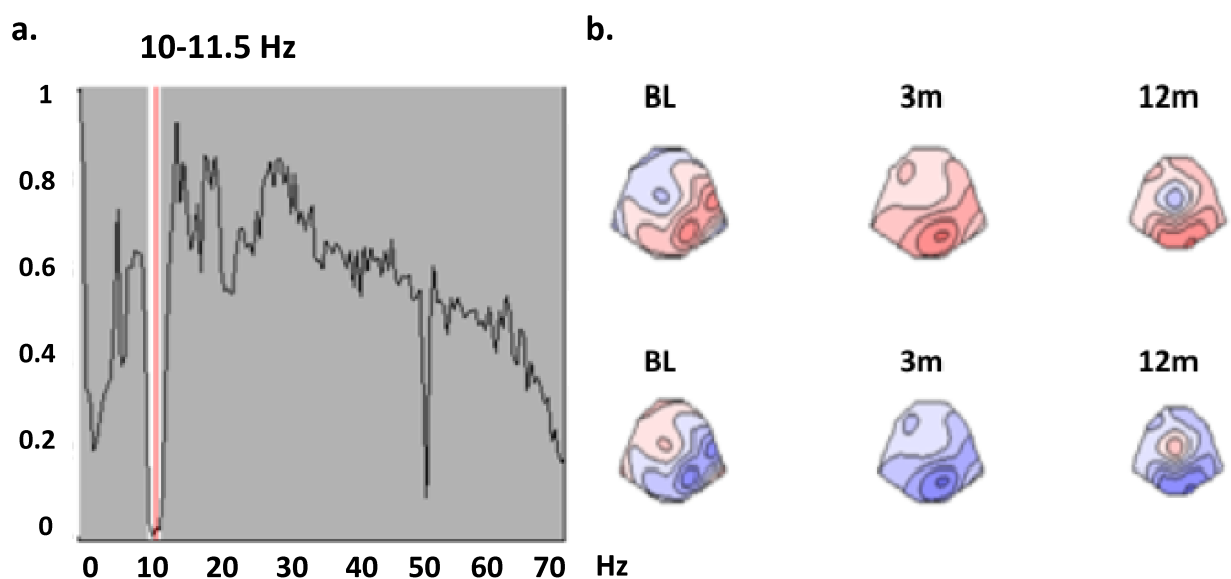
4.4.1 Stable or increased MMSE following NGF delivery correlated with an increase in alpha power

Using the Ragu software, we looked for possible changes in absolute power in the continuous frequency range from 0 to 70 Hz, and whether those changes correlated with changes between the end of NGF delivery and baseline, across the three time points of

EEG observations (baseline, 3 or 12 months) during the NGF delivery. The results are presented as topographic analysis of covariance (TANCOVA) maps of qEEG spectral power and correlation to MMSE changes over time. We found a significant correlation between alpha power at around 11 Hz (10-11.5 Hz) and changes from baseline in MMSE, i.e. less change in MMSE by the end of the study correlated with an increase in a narrow band of fast alpha power. QEEG at 3 months compared to baseline was marginally significant (0.059) while baseline against 12 months ($p=0.033$) and baseline against merged 3 and 12 months ($p=0.023$) showed a clearer, statistically significant trend. Interestingly, no additional change was observed between 3 and 12 months ($p=0.56$).

Figure 12. TANCOVA interaction between change from baseline in MMSE and EEG alpha power in the alpha frequency range, at **10-11.5 Hz**.

- Probability, p (y-axis) of the null hypothesis for a TANCOVA correlation between time and change from baseline (BL) in MMSE, as a function of frequency in Hz (x-axis). Significant ($p<0.05$) frequency range is marked by a white line.
- Topographic maps of the correlations between change from baseline (BL) in MMSE for the EEG observations at 3 months (3m) and 12 months (12m). Red maps display correlations between less than average change from baseline in MMSE score (less decline) and increase in alpha power in the 10-11.5 Hz frequency range. Blue maps display correlations between higher than average change from baseline in MMSE score (more decline) and a decrease in alpha power in the respective frequency range.



4.4.2 Increased CSF ChAT activity correlated with decrease in theta and increase in alpha power following NGF delivery

In the theta frequency band, at a frequency between 6-6.5 Hz, we found a weak correlation between theta power in EEG and less than average change from baseline in CSF ChAT activity (12 months minus baseline). This indicates that the more theta power, the less the increase in ChAT activity, but the correlation was only significant when 3 and 12-months were merged, compared to baseline ($p = 0.023$).

Furthermore, we found a correlation between the positive change from baseline in CSF ChAT activity at 12 months and increased power in the alpha power band at 11 – 11.5 Hz. The association was most prominent at 3 months ($p = 0.042$), indicating that at 3 months, the patients exhibited more fast alpha power associated with an increase in ChAT activity and this effect on EEG was marginally prolonged to 12 months ($p = 0.056$).

Here, for the first time, we demonstrate a relation between the cholinergic marker ChAT and EEG activity, particularly in the narrow fast alpha band during NGF delivery to the CBF in six AD patients. Nucleus basalis of Meynert is a major source of cholinergic projections to the cortex and hippocampus, and a reduction in the cholinergic markers ChAT and AChE in the AD brain, reflects the loss of cholinergic innervation (248). Particularly, ChAT activity has been used as a marker of cholinergic neuronal loss in lesion studies (249, 250). In animal models with nucleus basalis of Meynert lesions, EEG changes have been reported, with an increased power in the low frequency bands and decreased power in the high frequency bands, paralleled by decreased cortical activity of ChAT (251). ChEI treatment has repeatedly been shown to increase fast EEG activity in the alpha band, particularly in the posterior regions of the brain (252, 253), and this effect correlates well with improved cognition, measured with MMSE (253). Even cholinergic stimulation of non-demented subjects, with the ChEI galantamine results in increased alpha power and improved working memory performance (254). Our results indicate an association between changes in ChAT, a marker of cholinergic activity, and EEG activity, both in the slow theta and the fast alpha frequencies, mainly though during the first three months of NGF delivery. The patients had been treated stably with ChEIs for at least eight months prior to enrollment, which makes it less likely that the ChEI treatment would explain the observed association between the cognitive stabilization and cholinergic stimulation and increased alpha power in EEG during the study. While an earlier study on ChEI treated AD patients showed limited improvements of qEEG parameters for up to 6 months (252), the present study showed sustained association between a cognitive stabilization on MMSE and alpha power during NGF delivery. There was however no significant association between theta power and MMSE change, as was demonstrated for alpha power. Theta power increases early on in AD and thereafter reaches a plateau during the course of the disease (255, 256). Theta power may therefore not be used as a single electrophysiological marker for

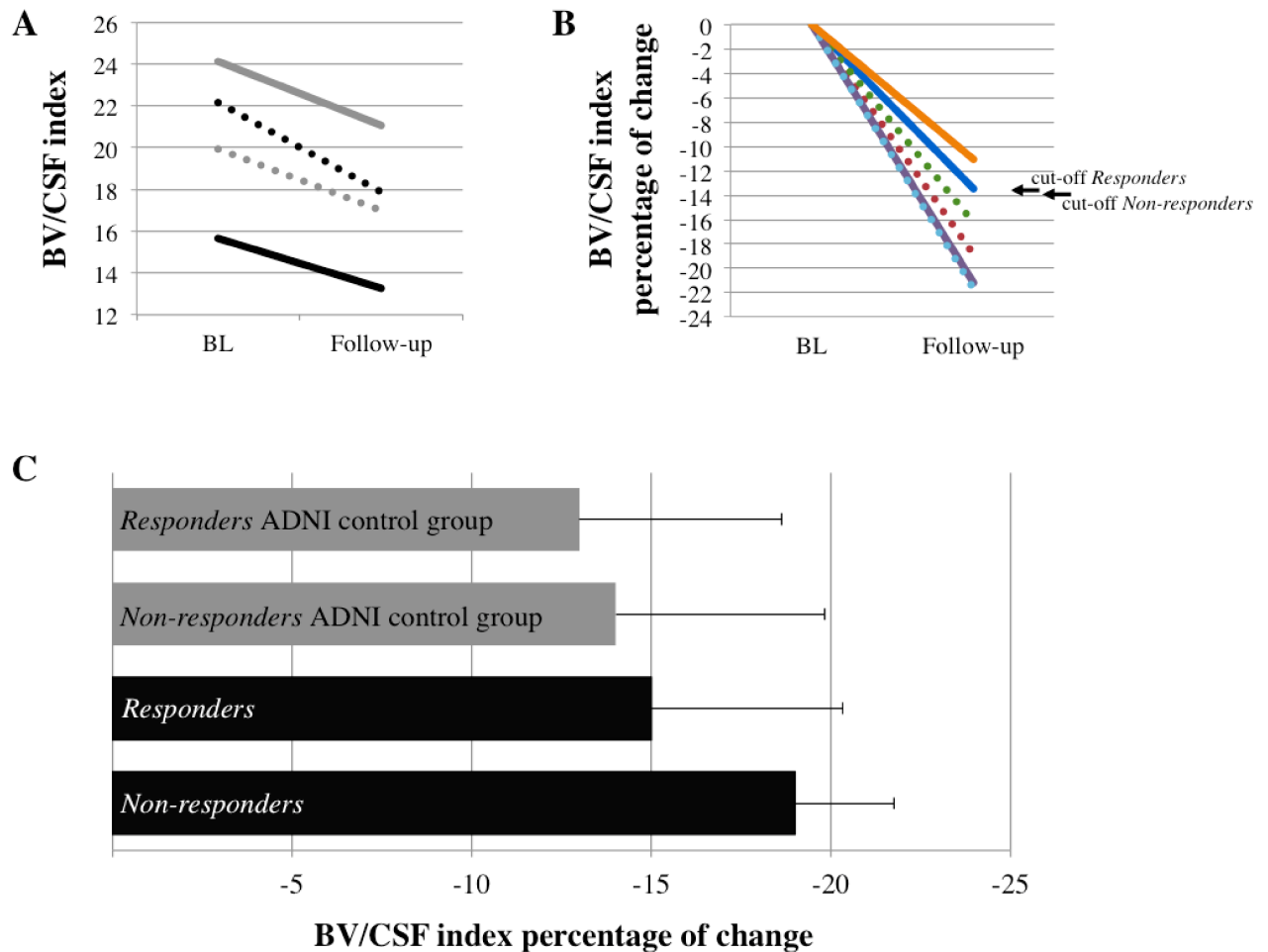
monitoring disease activity and therapeutic effect of stimulation of the cholinergic system, but should rather be used as an indicator of baseline disease severity.

4.5 STRUCTURAL BRAIN CHANGES ON MRI FOLLOWING NGF DELIVERY (PAPER IV)

4.5.1 Changes in brain atrophy following NGF delivery

At baseline of the study, the NGF *responders* (using the same definition of *responders* and *non-responders* as in **paper II**) exhibited a lower BV/CSF index (15.6 ± 0.1) than the *non-responders* (22.2 ± 8.2), while the 36 ADNI controls showed a lower BV/CSF index in the *non-responder* group (19.9 ± 7.5) as compared to *responders* (24.1 ± 11.3), indicating a higher degree of brain atrophy for the NGF *responders* at baseline as compared to *non-responders*. The NGF *responders* however showed a slower decline in brain atrophy during the 12 months of NGF delivery than the *non-responders*, while for the ADNI controls, there was no significant difference between the corresponding groups at the 12 month observation. Mean BV/CSF index for the NGF cohort was $18.9 (\pm 6.30)$ at baseline and $15.6 (\pm 4.67)$ at follow-up at 12 months and the difference was statistically significant ($p = 0.028$). For the ADNI controls, mean BV/CSF index also decreased significantly between baseline and 12 months. We found no difference in hippocampal volume between the NGF *responders* and *non-responders*.

Figure 13. A. Means for the following groups at two time points, baseline (BL) and 12-months (Follow-up): NGF cohort (black) and ADNI cohort (gray), *responders* in both cohorts are displayed in solid lines, *non-responders* in dotted lines. *B.* Change from baseline for individual NGF patient, *responders* are displayed in solid lines, *non-responders* in dotted lines. The arrows show normative cut-offs calculated from *responders* and *non-responders* in the ADNI cohort. *C.* NGF *responders* showed slower decline than NGF *non-responders*, while the corresponding ADNI groups showed a similar decline in brain atrophy during the follow-up period. Reprinted from Ferreira et al, 2015 with permission from IOS press (257).



4.5.2 Stabilization of brain atrophy following NGF delivery correlated with clinical outcome measures

Upon follow-up at 12 months, the NGF *responders* showed less decline on outcome measures, such as CDR sum of boxes, ADAS-Cog, CSF A β and on the Instrumental activities of daily living (IADL) scale (258). They also showed a less increase in NFL than the *non-responder* group but they also exhibited more increase in CSF t-tau. Baseline BV/CSF index correlated significantly with longitudinal changes in CSF A β_{42} ($r = -0.943$, $p = 0.005$), indicating that patients with greater brain atrophy at baseline showed an improvement in CSF A β_{42} at follow-up.

At baseline, the NGF *responders* exhibited a higher degree of neuronal degeneration, indicated by more brain atrophy and higher t-tau. Interestingly, this group showed a more favorable clinical progression and less progression of brain atrophy at the end of the study as well as stable levels of CSF A β_{42} and t-tau. The fact that the *responder* group exhibited a higher degree of neurodegeneration at the start of the study and at the same time showed a more favorable overall clinical status at the end of the study may seem

contradictory. In the literature, the differentiation between clinical and pathophysiological severity has been attributed to cognitive reserve (259), or the ability to endure brain injury without showing a corresponding degree of clinical symptoms. We hypothesize that the *responder* patients may have had a higher degree of cognitive reserve but this must be studied further and additional proxies of cognitive reserve, e.g. social and intellectual engagements and brain reserve (status of functional brain networks) may be of interest to further test this hypothesis.

At the end of the study, the *responder* group showed lower progress in brain atrophy, less worsening in clinical parameters as well as less pronounced changes in CSF biomarkers which may further support this hypothesis of cognitive reserve. The NGF *responders* showed a similar degree of hippocampal atrophy as the *non-responders* over time, suggesting a more generalized response to NGF delivery in the brain involving wide cerebral projections, rather than a focal response in hippocampal projections.

Results obtained from automatic MRI segmentation (as applied in this study), have the advantage of being free from rater bias, placebo effect or chance and are thus used increasingly as outcome measures in clinical trials (260). We furthermore propose the usefulness of the BV/CSF index as a tool for monitoring longitudinal changes in brain atrophy in clinical trials where a marker of brain atrophy is needed. To our knowledge, this is **the first study** reporting longitudinal changes in atrophy parameters in patients receiving NGF delivery to the basal forebrain. Due to the small cohort size, the results must be interpreted with caution and confirmed in larger studies.

4.6 ENCAPSULATED CELL BIODELIVERY OF NGF TO THE BASAL FOREBRAIN IN FOUR AD PATIENTS, A DOSE ESCALATION STEP (PAPER V)

4.6.1 Patient demographics and diagnosis of AD

Four patients were enrolled to this third dose step of the NGF-ECB trial with a duration of six months and demographic data are presented in *table 3*. The patients were treated with a stable ChEI treatment for a median duration of 17 months prior to enrollment, and remained on ChEI treatment throughout the study. All four patients had well-controlled hypertension and were normotensive at enrollment. Three of four patients had a history of mild to moderate depression and one patient was treated for hypothyroidism. Brain glucose utilization, assessed with FDG-PET at baseline, was consistent with AD and histology by cortical biopsies taken from the implant site confirmed the diagnosis of AD in all four patients.

Table 3. Demographic and CSF data on enrolled patients, at baseline, 3 and 6 months.

	Baseline median (range)	3 months median (range)	6 months median (range)
Gender, n (M/F)	3M /1F		
Age (years)	63.5 (57-68)		
Memory problems (years)	4 (0.5-8)		
Time from AD diagnosis (years)	1.5 (0.5-5)		
Duration of ChEI (months)	1.4 (0.5-5)		
MMSE score	21 (16-23)	18 (15-24)	19 (14-21)
ADAS-Cog score	29 (23-35)	37 (21-46)	33 (26-41)
CDR score (sum of boxes)	0.75 (0.5-1)	1 (0.5-1)	1 (1-2)
IADL score	19 (17-21)	17 (15-23)	23 (14-24)
CSF A β ₄₂ (ng/L)	188 (134-208)	202 (113-207)	183 (123-206)
CSF t-tau (ng/L)	128 (70-235)	120 (64-256)	113 (76-187)
CSF p-tau (ng/L)	36 (18-76)	42 (35-48)	46 (33-53)
CSF NFL (ng/L)	301 (57-557)	1959 (846-2632)	718 (236-758)
CSF GFAP (ng/L)	586 (395-891)	612 (387-943)	589 (535-833)
CSF ChAT activity (nmol/min/mL)	374 (317-447)	367 (279-414)	358 (337-410)
CSF AChE activity (nmol/min/mL)	12 (9-22)	10 (8.6-20.9)	10 (9-23)

4.6.2 Safety and tolerability

All four patients underwent stereotactic implantations of the second-generation NGF-ECB implants, without immediate surgical complications. Cranial CT scans were performed post-operatively to confirm implant positions, showing no adverse events. The scalp incisions healed well in all patients, and the postoperative course was mainly uneventful. 16 adverse events (AEs) were reported during the study but no serious adverse event. One AE was of medical importance though, a left-side parieto-occipital subacute subdural hematoma (SDH), discovered incidentally at the 3-month MRI follow-up, without a midline shift, and in no connection to the NGF-ECB implants. It's important to note, that one patient in the first cohort of the NGF-ECB trial suffered a SDH, also incidentally discovered at the 3-month MRI follow-up, and had resolved within 3 months (176). Both these SDHs gave no or very mild symptoms of fatigue. They were treated conservatively and resolved completely. They may be related to the previous surgery in combination with brain atrophy. None of the patients showed any sign of weight loss or back pain. The most

common AEs were a mild post-operative headache and transient post-operative delirium. All AEs were resolved at the end of the trial and all four patients could complete the study.

4.6.3 Implant performance

Pre-operative release of NGF for the sixteen NsG0202.1 implants selected for implanting was on average 8.6 ± 2.2 ng NGF/24 hours, and was thus five times higher than NGF release of the first-generation NGF implant, NsG002 (177). Upon retrieval of the implants at the end of this dose step, all implants were proven to be intact, and no inflammation or connective tissue was found. 13 out of 16 implants released NGF and 48 hours after retrieval, the average NGF released per implant/24 hours was 6.1 ng, 5.9 ng, 6.8 ng and 37.2 ng.

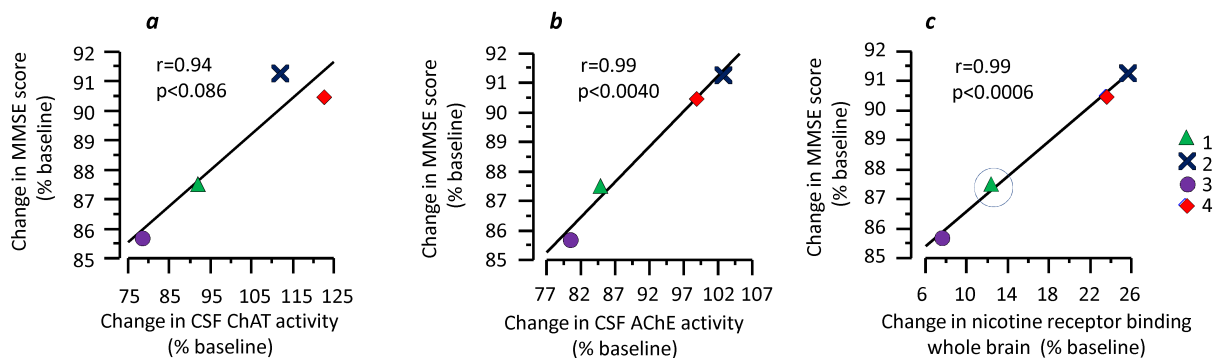
As a result of dissatisfactory gene expression and low cell survival at retrieval for the first-generation implant, we developed this second-generation NGF-ECB implant, NsG0202.1 for a dose-escalation step. The implant and cell line were subjected to modifications, including optimization of the plastic material in the implant, providing better cell support and improved manufacturability and application the Sleeping Beauty (SB) transposon expression system (261) to achieve a higher secretion of NGF. The SB gene vector system is a non-viral system, able to provide an efficient and stable gene transfer with long-term gene expression (262) and this study is **the first to report a clinical application of SB transposon-mediated gene delivery** to the central nervous system in man.

4.6.4 Secondary outcome measures

All four patients showed a decline in cognitive performance on MMSE by 2-3 points and an increase in ADAS-Cog score (inverse scale, the higher the score, the worse cognitive performance) during the 6-month trial period, paralleled by a decrease in BV/CSF index on MRI from baseline.

Further analyses revealed no significant differences in the CSF AD biomarkers, as compared to baseline. Levels CSF NFL increased at the 3-month observation point, but had decreased at 6 months, albeit not to baseline levels. Activities of CSF ChAT and AChE were increased in two patients at 6 months and this increase correlated with a more stable cognitive performance (less decline in MMSE) at the end of the trial step.

Figure 14. Correlations between changes from baseline in MMSE and CSF cholinergic markers (*a* & *b*), and nicotine receptor binding in the whole brain (*c*). The baseline value for nicotine receptor binding for patient marked number 1 was not available (marked with a circle), due to technical difficulties with the PET tracer and for the purpose of calculating change from baseline, we extrapolated an average of baseline values from the other three patients. Reprinted with modifications from Eyjolfssdottir et al 2016, with permission from BioMed Central (210).



We observed no change in ^{11}C nicotine receptor binding by PET during the 6-month trial step. Longitudinal change in whole brain ^{11}C nicotine binding did though correlate with change from baseline in MMSE (fig. 14 *c*).

Results from previous NGF delivery trials have, in a subset of patients, shown improved cognitive performance (172), brain glucose utilization and nicotine receptor binding on PET as well as improved EEG pattern (164, 165). In this dose step, we found no evidence of improved cognition during the 6 months, as was expected considering the short duration of the trial step, but neither were there signs of a greater cognitive decline than was expected during 6 months according to previous studies (225). There was no evidence from our data for changes in levels of the CSF biomarkers. NFL levels were though increased at the 3-month observation and although they had decreased somewhat at 6 months, the levels were still increased compared to baseline and this was also reported in the previous clinical trial with NGF-ECB (176). This has been interpreted as a sign of a temporal neuronal damage related to the neurosurgery. NFL is a marker of acute neuronal damage (263) and a similar observation was also reported from a study of deep brain stimulation electrode implantation in Parkinson's disease patients, declining over a few months (264). Levels of GFAP, a marker of glial cell activation after damage, were however unaffected.

In accordance with our previous findings, we found correlations between changes from baseline in MMSE and changes in three markers of cholinergic activity, the activities of the ACh synthesizer ChAT and the ACh degrading enzyme AChE and nicotine receptor binding measured by PET. There were no changes on atrophy parameters measured by MRI in this dose cohort, but this was not to be expected considering the short trial period.

4.7 STRENGTHS AND LIMITATIONS

Encapsulated cell biodelivery has advanced as a therapeutic option for neurodegenerative disorders. This technology has several advantages from a safety perspective. The cells being encapsulated provides immunoisolation reducing risk for immunoreactions, and the technique also makes removal of the cells possible in case of complication or replacement of the implant with new or modified cells without the use of stereotactic surgery (213). Moreover, there is no genetic modification of the patients' own cells, diminishing risk of mutations or carcinogenesis.

The NGF-ECB study has several limitations, the most significant being the invasiveness of the surgical procedure, which induces a strategic brain lesion during implantation. The method is still at an early experimental stage, and poses heavy regulatory, ethical and economical demands on the investigators, which explains the small number of patients included. This in turn limits the use of conventional statistics so that interpretation of the data calls for due caution. As a partial countermeasure for this limitation, control groups were included for data comparisons in two studies from this group (**papers II & IV**). Another limitation in statistical analyses (**in papers II & III**), is that no corrections for multiple comparisons were done due to the exploratory nature of the studies, but as a compensation we weighted the overall patterns of observations rather than emphasizing isolated findings. An additional limitation is that there was no possibility to determine the NGF release *in vivo* in the brain, making it impossible to relate changes in cognition and biomarkers to the *in vivo* release of NGF, until the NGF-ECB implants were retrieved.

5 CONCLUDING REMARKS

The **main aim of this thesis** was to explore the potential of encapsulated cell biodelivery of NGF with regard to safety, tolerability and possible disease-modifying effects in a total of ten AD patients. A secondary aim was to explore the levels of neurotrophins and their respective pro-peptides in CSF.

In **papers II-IV**, effects of NGF delivery using a NGF-ECB implant, in a **first in man study** in six AD patients, were explored on cholinergic markers in CSF, EEG spectral power and spatial distribution and atrophy on MRI. In **paper V**, safety, tolerability and effects on biomarkers were explored for a second-generation NGF-ECB implant, targeted to the cholinergic basal forebrain in four AD patients. In **paper I**, we investigated levels of the neurotrophins NGF and BDNF and their respective pro-forms in CSF samples from subjects with AD, compared to subjects with MCI and SCI.

In our studies on biological effects of NGF delivery, the results point towards a stimulation of the cholinergic system, as measured by an increase in the activities of the cholinergic markers ChAT and AChE in CSF, a favorable change in EEG power in the fast alpha spectrum, and a slowing rate of brain atrophy in a subset of patients. Our studies on CSF levels of neurotrophins our results indicate an alteration in the levels of neurotrophin pro-forms in MCI.

The key findings of this thesis may be summarized as follows:

- safety and tolerability was demonstrated for both NGF-ECB implants and no adverse events in all three dose cohorts were deemed to be related to NGF delivery or the ECB-NGF device,
- the second-generation NGF-ECB implant NsG0202.1 showed a sustained release of NGF in a majority of implants and released five times higher levels of NGF than the first-generation NsG0202 implant,
- the cholinergic marker ChAT, recently demonstrated to be present extracellularly in CSF, may be a useful biomarker for monitoring treatment effect on the cholinergic system,
- the results suggest, for the first time, a pattern of associations between cholinergic markers in CSF, suggestive of beneficial effects of NGF delivery, supporting a proof-of-principle effect,
- increase in power in the narrow fast alpha frequency band in EEG was associated with a less decline in MMSE and an increase in ChAT activity following NGF delivery, in particular after the first three months of treatment, further supporting observed modifications of the cholinergic system,

- less cognitive decline was associated with less brain atrophy following NGF delivery in a subset of patients, and the observed slower rate of brain atrophy was also associated with favorable changes in clinical outcome measures,
- changes in neurotrophin pro-peptides may be predictive of a progress in cognitive decline in subjects with MCI.

6 FUTURE RESEARCH DIRECTIONS

There is convincing evidence supporting the role of cholinergic dysfunction as one of the pathophysiological events leading to the development of AD. The importance of NGF for the growth and survival of cholinergic basal forebrain neurons is also well documented. There is thus great support for the implication of NGF delivery as a potential disease-modifying therapeutic strategy in this neurodegenerative disease. There is, however, a huge caveat in that the NGF molecule does not pass the blood-brain barrier, and that it cannot be administered systemically to the central nervous system due to side-effects.

Extensive testing with NGF delivery to the cholinergic basal forebrain in animals prior to human trials have demonstrated safety, tolerability and possible therapeutic effects. A handful of trials have now been conducted but they have all been limited by the invasiveness of the stereotactic neurosurgery and by the risks of general anesthesia in this sensitive patient group. The patients must therefore be selected carefully with regard to age and comorbidities.

With this said, it would be very attractive to be able to develop a non-invasive way of NGF administration without the side-effects of systemic administration, and this is indeed under current investigation in animals (265) in the form of intranasal delivery of NGF.

As for future development of the NGF-ECB technique, the implant design is under further refinement to obtain an even more predictable and stable delivery. For a phase II study testing effects on biomarkers and clinical outcome measures, a trial on a larger cohort, with a longer duration, and preferably with a sham-operated control group for comparison, or at least a control group of ChEI-treated AD patients, monitored closely with regard to the same outcome measures, would be desirable. Our overall results indicate that AD patients enrolled in such a trial will optimally need to have a rather stable cognitive performance prior to enrollment and furthermore, have a mild degree of brain atrophy, to reduce safety risks of the surgical procedure.

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